Secondary bile acids effects in colon pathology. Experimental mice study[1]

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ABSTRACT

PURPOSE: To assess whether deoxycholic acid (DOC) and lithocholic acid (LCA) administered in a period of six months in a concentration of 0.25% may have a carcinogenic role in mice colon.

METHODS: The study used C57BL6 female mice divided into four groups. The control group received a balanced diet and the others received diets supplemented with 0.25% DOC, 0.25% LCA and 0.125% DOC+0.125% LCA, respectively. After euthanasia, the lesions found in the resected gastrointestinal tracts were stained with hematoxylin-eosin and examined microscopically.

RESULTS: No gastrointestinal tract changes were observed in the control group, while hyperplastic Peyer's patches in the small intestine, flat adenomas with mild dysplasia and chronic colitis at the level of the colon were found in all three test groups. The colonic lesions prevailed in the proximal colon. The highest number of flat adenoma lesions (8), hyperplasia of Peyer's patches (25) and chronic colitis (2) were found in mice fed with diet and LCA.

CONCLUSION: Precancerous or cancerous pathological lesions could not be identified. Instead, adenomatous colonic injuries occurred in a shorter period of time (six months), compared to the reported data.

Key words: Deoxycholic Acid. Lithocholic Acid. Colon. Mice.

Introduction

Synthesis of primary bile acids, cholic and chenodeoxycholic, occurs in hepatocytes by oxidizing cholesterol in a multi-gradual process. Thus they become secondary bile acids, deoxycholic acid (formed from colic acid) and lithocholic acid (formed from chenodeoxycholic acid), only when they are released in the intestine via the bile ducts and under the action of bacteria. Therefore these four bile acids (primary and secondary) can be absorbed into the bloodstream from the intestine, returned to the liver and re-secreted, thereby establishing virtually an enterohepatic circulation[1,2]. Carcinogenesis takes place in three distinct phases: initiation, promotion and progression. An agent is considered carcinogenic when it is present in all these stages. A promoter cannot initiate the process, although it can participate in the other stages, but only if a carcinogenic agent co-exists and has initiated the process[3].

The bile acids activity in carcinogenesis and its multiple mechanisms have been described in detail by Bernstein *et al*.[4]. Deoxycholic acid and lithocholic acid seem to be, as secondary bile acids, the most significant agents in the etiology of colorectal cancer in humans[5].

A high-fat and high-beef diet leads to increased excretion of secondary bile acids, especially deoxy and lithocholic acids in feces[6]. High levels of bile acids due to high–fat diets (individuals with a Western-style diet) may act as carcinogens in the development of colon cancer in humans[7]. The function as carcinogenic promoters of these secondary bile acids has been demonstrated in numerous studies, including those of Baijal *et al*.[8], Seraj *et al*.[9], Narisawa *et al*.[10], Reddy *et al*.[11], Narahari *et al*.[12] and many others, by experiments in which these acids were administrated to lab animals (mice, rats) previously treated with a proved carcinogen. In these conditions, the reports revealed an increased multiplication of ACF (aberrant crypt focus) or an increased in number or size of tumors occurred due to the action of the carcinogen factor, compared with the absence of such changes in the control groups (not pretreated with carcinogen). Moreover, Bernstein *et al*.[7] published an article to prove the role of deoxycholic acid as carcinogen agent when is administred orally to mice for 8-10 months in an increased concentration of 0.2 %. They proved thus that the presence of this bile acid in the gastrointestinal tract over a long period of time can lead to the occurrence of adenomas and colon carcinomas in mice.

Considering that both deoxycholic acid (DOC) and lithocholic acid (LCA) can also be found associated in the gastrointestinal (GI) tract of humans, our study aims to assess whether DOC, LCA or their association (DOC/LCA) may have carcinogenic role in mice colon when a slightly higher concentration (0.25%) is added in the daily diet over a shorter period of time (six months). The effects will be evaluated also by examining both the injuries eventually occurred in all other segments of the lower GI tract and the possible distant lesions.

Methods

The research infrastructure was provided by the University of Medicine and Pharmacy of Tirgu-Mures, Romania, including facilities of the University Experimental Station. The study was conducted under the Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Directive 2010/63/EU of the European Parliament and the Council on the protection of animals used for scientific purposes, Annex III, 22 September 2010) and it was approved by the Research Ethics Committee of the University (no. 43/2.07.2014).

A homogeneous group of 28 female lab mice from the C57BL6 species (Cantacuzino Institute, Bucharest, Romania) were used and kept according to EU guidelines: in special cages, in a 12-h cycle, appropriate microclimate, having free access to water (*ad libitum*). The mice were acquired at an age of four to six weeks, free of any specifically pathogens and they were monitored weekly in terms of general health, the amount of granulated food consumed per day and their weight curve.

Basic diet provided to mice

A normal caloric balanced diet was administered daily to mice, since their birth until the sacrifice, and consisted in a granulated food in form of very hard pellets manufactured by hot pressing, a homogenized mixture of: ground grains, soybean meal, sunflower meal, calcium carbonate, monocalcium phosphate, amino acids and premixed vitamin - minerals (Table 1). This composition satisfies the necessary for maintenance and reproductions according to NRC Nutrient Requirement of Laboratory Animals (National Academy of Sciences: Guide for the Care and Use of Laboratory Animals, National Academies Press, Washington, D.C).

TABLE 1 - Composition of granulated food used as basic diet (according to technical specification provided by manufacturer).

No.	Nutrient	Mouse daily intake / feed unit (10% moisture)
1.	Digestible energy (KJ/g): (*Metabolized energy* ≈ 90-95 % of the digestible energy)	**16.0**
2.	Fat (mg/g):	**50**
3.	Raw fibers (no necessary):	**n.n.**
4.	Raw proteins (mg/g):	**150**
5.	**Amino Acids (mg/g):** Arginine (4.3), Asparagine (4), Glutamic acid (40), Histidine (2.8), Isoleucine (6.2), Leucine (10.7), Lysine (9.2), Methionine and Cysteine (9.8), Phenylalanine and Tyrosine (10.2), Threonine (6.2), Tryptophan (2), Valine (7.4).	**112.8**
6.	**Minerals (mg/g):** Calcium (5), Magnesium (0.5), Chloride (0.5), Phosphor (3), Potassium (3.6), Sodium (0.5), Cooper (5), Iodine (0.15), Iron (35), Selenium (0.15).	**53.4**
7.	**Vitamins (mg/g):** Thiamin -B_1 (4), Riboflavin -B_2 (3), Pyridoxine -B_6 (6), Cyanocobalmin -B_{12} (50), Menadione –K (1), Retinol –A (1.2), Cholecalciferol -D_3 (25), Tocopherol –E (27), Ascorbic Acid –C (n.n.), Choline (750).	**867.2**

Protocol of the experimentally study

After a quarantine of two weeks (the start of experiment): The mice were divided into four groups (seven individuals/ group), each group being then fed with its specific diet on the each day of the experimental period.

After 140 days (the measurement of the bile acids levels in feces): The feces from five mice of each group were harvested (preventing coprophagia), three consecutively days. The collected material was then subjected to the secondary bile acids levels determination.

After six months (the end of experiment): All mice were sedated by intraperitoneally injection of a mixture of Ketamine (80 mg/ kg)/ Xylazine (10 mg/kg,), underwent the extensive resection of the GI tract and finally, were euthanized by intracardiac injection of T61 (mixture of Embutramide/ Tetracaine hydrochloride/ Mebezonium iodide), in dose of 0.05 ml/mouse. The collected biological material was then subjected to the histopathological examination.

Specific daily diets prepared for the mice groups

Specific ingredients used: Deoxycholic Acid, 99% purity (Alfa Aesar, product no.820061, lot no.10179328), Lithocholic Acid, 97% purity (Sigma Aldrich, product no.L6250, lot no.BCBN2648V), Gelatin (UTCHIM, Romania).

Daily content of the specific diets: The control group (group 1 = C) received on all period of the actual testing (six months) a normal caloric balanced diet supplemented with 0.5% gelatin (alimentary grade; it was necessary as binder agent in the process of the supplemented bile acids diets preparation). The testing groups received in addition to 0.5% gelatin, secondary bile acids, as it follows: DOC 0.25% (group 2 = T_{DOC}), LCA 0.25% (group 3 = T_{LCA}) and 0.125% DOC + 0.125% LCA (group 4 = $T_{DOC/LCA}$).

Quantification of secondary bile acids in the mice feces

The analytical method: The deoxycholic and litocholic acid content in mouse feces were determined by a validated high-throughput liquid chromatography (HPLC)/mass spectrometry method. The HPLC system was an Agilent 1100 series (binary pump, autosampler, thermostat; Agilent Technologies, Santa Clara, California), coupled with a Brucker Ion Trap SL (Brucker Daltonics GmbH, Leipzig, Germany). A Zorbax SB-C18 chromatographic column (100 × 3.0 mm i.d., 3.5 µm; Agilent Technologies) was used. The mobile phase consisted of 55:45 (V/V) water/acetonitrile. The flow rate was 1 mL/min, and the thermostat temperature set at 45°C. The mass spectrometry detection was in multiple-reaction monitoring mode for DOC (m/z 391.4→m/z 345.4) and single ion monitoring mode for LCA (m/z 375.3), negative ions, using an atmospheric pressure chemical ionization source. The retention times were 1.4 min for DOC and 2.3 min for LCA, respectively.

The biologic samples (n=3) were prepared as follows: After fine grounding of mouse feces, a sample of 0.5 g was weighed and about 15 ml methanol were added. The mixture is

kept for 10 min in an ultrasonic bath, then filtered and brought to 25 ml in a volumetric flash by washing the residue. A volume of 1 µL was injected into the liquid chromatography/mass spectrometry system.

The calibration curves for DOC and LCA: Were linear in concentration range of 0.6 to 20 µg/mL, with a correlation coefficient r greater than 0.991.

Histopathological examination of mice GI tracts

Digestive tube from the jejunum to the anal canal was resected en bloc, cut longitudinally throughout, photographed and subjected to macroscopic and microscopic examinations. It was also possible to search thoroughly for distant metastases through peritoneal cavity and organs. After sectioning different segments of the digestive tract, they were photographed with a Samsung 16 megapixel camera and fixed in 4% formalin solution.

Fragments of the gut changes, previously fixed in formaldehyde, were sampled and embedded in paraffin, sectioned with a rotary microtome and stained with hematoxylin and eosin. The prepared fragments thus obtained were examined with a Nikon Eclipse E800 microscope equipped with digital photographic camera.

Statistical analysis of experimental results

Standard deviation (SD) was calculated in the case of the results determined by the assessment of bile acids in the mice feces. Student t-test was applied for analysis the same lot and also the amount of diet consumed by the mice, while Anova and Bonferroni tests were applied to compare the variability between the mice lots. Chi-square test was used for the histopathological lesions analysis. In all cases, the level of statistical significance was set at $p<0.05$.

Results

The monitored daily diets parameters and the levels of bile acids found in the mice feces

In the experimental study, four groups of mice were fed with special diets for a period of six months, as it follows: a control group (group 1 = C), DOC 0.25% (group 2 = T_{DOC}), LCA 0.25% (group 3 = T_{LCA}) and 0.125% DOC + 0.125% LCA (group 4 = $T_{DOC/LCA}$). During the experimental period, the mice were monitored weekly in terms of general health, the amount of granulated food consumed per day, their weight curve and the bile acids levels excreted in the feces (Table 2).

TABLE 2 - Parameters determined/calculated during the 6 months of experimental study in mice.

Mice groups / Investigated bile acids		**1. C (control)**		**2. T_{DOC}**		**3. T_{LCA}**		**4. $T_{DOC/LCA}$**		***Experimental remarks:***
		DOC	**LCA**	**DOC**	**LCA**	**DOC**	**LCA**	**DOC**	**LCA**	*Amount of food consumed (g/mouse):*
Added in food (mg/g)		-	-	2500	-	-	2500	1250	1250	
Calculated daily intake	mg/ mouse	-	-	6.52 – 7.30	-	-	6.52 – 7.30	3.26 – 3.65	3.26 – 3.65	2.61 CI = 2.50 - 2.70 (at onset) – 2.92 CI = 2.85 - 3.11 (at the end)
onset – end	mg/g of body weight (= mg/ kg)	-	-	0.375 – 0.303	-	-	0.334 – 0.317	0.189 – 0.153	0.189 – 0.153	
Assessed in dried feces, after 140 days (mg/g ± SD)		**0.16** ± 0.069	**0.02** ± 0.003	**1.85** ± 0.314	0	**0.12** ± 0.022	**4.66** ± 0.852	**1.16** ± 0.153	**1.25** ± 0.335	*Significance:* **p = 0.002*** (Anova test)
		Mouse weight variation:								*Significance:*
-at onset (g)		18.9		17.4		19.2		17.2		**® p = 0.24****
-at the end (g)		23.8		24.1		23.0		23.8		**® p = 0.85****
Significance (t-test):		p = 0.0001*		p = 0.0001*		p = 0.09**		p = 0.003*		(Anova test)

*statistically significant ($p<0.05$); ** statistically insignificant ($p>0.05$); CI = confidence interval.
Deoxicholic acid (DCA),Lithocolic acid (LCA)

The good general condition of the mice has maintained since the onset till the end of experiment, with no deaths, but with increased diuresis in groups 3 (T_{LCA}) and 4 ($T_{DOC/LCA}$) compared with the control group (C) and the group 2 (T_{DOC}). At the onset of the experiment, the groups were relatively homogeneous with no statistical significant differences (p = 0.24) and in each group the mice significantly gained their weight (the end vs. the onset). The initial homogeneity between the groups has maintained until the end (p = 0.85). The amount of food consumed by the mice averaged 2.61 g/mice (confidence interval (CI) = 2.5 g – 2.7 g) between groups in the first part of the study and reached to 2.92 g/mice (CI = 2.85 g - 3.11 g) in the last weeks (before euthanasia), the difference being statistically significant (p = 0.002).

The histopathological changes found in mice GI tracts

Macroscopic pathological changes

Examining the biological material collected from the control group (C), changes in the GI tracts have not been seen nor in the peritoneal cavity. The discovered lesions found in the three groups of mice tested are presented in Table 3.

TABLE 3 - Macroscopic lesions produced by bile acids intake during six months.

Tested groups/ no. of mouse/ weight (g)			Type/ Number of changes found in the resected segments of mice gastrointestinal tracts					
			Peyer's patches		Flat adenoma		Chronic colitis	
			jejunum	*ileum**	*proximal colon***	*distal colon*	*proximal colon*	*distal colon*
TDOC	1	22	2	2	1	-	-	-
	2	25	1	-	1	-	-	-
	3	23	3	1	-	-	-	1
	4	26	-	-	-	-	-	-
	5	24	1	3	-	-	-	-
	6	26	3	1	-	-	-	1
	7	23	2	2	-	-	-	-
TLCA	1	27	2	4	2	-	-	-
	2	26	2	3	1	-	-	-
	3	23	2	3	1	-	-	1
	4	26	3		1	-	-	-
	5	22	2	1	2	-	-	-
	6	23	2	1	-	-	1	-
	7	14	-	-	1	-	-	-
TDOC/ LCA	1	27	2	-	-	-	-	-
	2	25	2	-	Inclusion fault			
	3	26	-	-	-	-	-	1
	4	22	-	-	1	-	-	-
	5	27	3	2	-	-	-	-
	6	18	3	1	Autolyzed colon			
	7	22	1	-	1	-	-	-

Statistically significant ($p<0.05$) by Chi-square test:
* p = 0.04 (T_{DOC} vs. $T_{DOC/LCA}$); p = 0.003 (T_{LCA} vs. $T_{DOC/LCA}$);
**p = 0.02 (T_{DOC} vs. T_{LCA}); p = 0.75 (T_{DOC} vs. $T_{DOC/LCA}$); p = 0.02 (T_{LCA} vs. $T_{DOC/LCA}$)
Deoxicholic acid (DCA),Lithocolic acid (LCA)

In the analysis of variance between the groups, the identified numbers of changes were found with statistical significance. Thus, the highest number of flat adenoma lesions in the proximal colon encountered in group T_{LCA} is statistical significant compared to groups T_{DOC} and $T_{DOC/LCA}$ (p = 0.02, both cases). The highest number of changes in all three tested groups consisted in hypertrophied Peyer's patches, their frequency in the jejunum being distributed with no statistically significance between the groups, but with statistical significance in ileum, with more frequent injuries in groups T_{DOC} and T_{LCA} compared to $T_{DOC/LCA}$ (p = 0.04, p = 0.003).

Microscopic histopathological confirmations

Flat adenoma with mild dysplasia

At the level of the colonic wall a flat/sessile lesion with broad base of implantation was noted. The folds appear thickened and elongated due to crypt hyperplasia. The latter are multiplied, regular, uniform and the glandular epithelium surrounding them show relatively uniform nuclei with minimum pleomorphism, slight hyperchromazia, sometimes crowded showing a stratification tendency. There was a slight depletion of mucus in areas affected by glandular epithelium dysplasia (Figure 1).

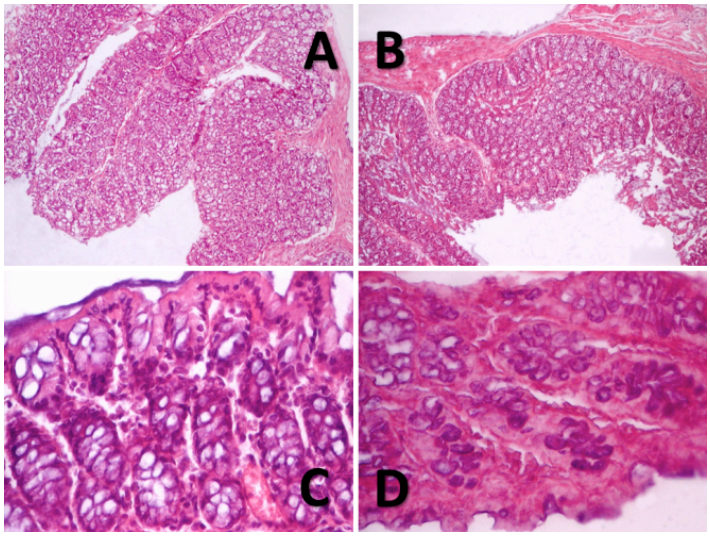

FIGURE 1 - Microscopic changes indicating a flat adenoma with mild dysplasia in the mice proximal colon: A, B-flat lesion with uniform, hyperplastic crypts, hematoxylin and eosin, x4; C-hyperchromic nuclei with low pleomorphism, and a slight tendency to stratification, hematoxylin and eosin, x20; D-dysplastic glandular epithelium with mucin depletion, hematoxylin and eosin, x20.

Lymphatic tissue hyperplasia with germinal center/Peyer's plate hyperplasia

In nearly the entire small bowel wall thickness from the surface to the subserous corion layer, a nodular lymphocyte infiltration was evidenced, consisting of mature uniform small lymphocytes. It was also found the presence of a germinal center.

Chronic colitis

In the entire thickness of the colonic wall corion edema was observed, accompanied by congested blood capillaries, along with a moderate mixed inflammatory infiltrate composed of lymphocytes, plasma cells and segmented granulocytes.

Discussion

Secondary bile acids in mice: pathological potential of the exposure on six months

The two secondary bile acids considered in this study were administered, over a period of six months, as supplements in the daily diet to mice in three concentrations: 0.25% DCA, 0.25% LCA and 0.25% mixture of DCA/LCA (1:1). The amount of food consumed by each mouse of the four mice groups has increased significantly ($p = 0.02$) as well as the average weight in three groups: the control group ($p = 0.0001$), group T_{DOC} ($p = 0.0001$) and group $T_{DOC/LCA}$ ($p = 0.03$) and insignificantly in group T_{LCA} ($p = 0.09$). Comparing the groups between them right before euthanasia, their weight can be considered as equal, with no significant differences, which shows that mice were fed relatively uniform, without excesses, the groups being homogeneous in this regard. This weight gain suggests that the appearance of the pathological formations identified in the GI tract of mice is due to the exogenous secondary bile acids daily intake. Their presence in the GI tract was confirmed by their increased levels excreted into feces in cases of the test groups compared to the control group. The changes evidenced in the small intestine in form of Peyer's plate hyperplasia appeared in a higher frequency in the jejunum compared to the ileum. Regarding the flat adenomas, these changes were found exclusively in the proximal colon, while chronic colitis was located more frequently in the distal colon (four in distal colon *vs*. one in proximal colon).

The changes found in this experimental study are overlapping with the literature reported data. Lesions location in the proximal colon is also reported by Bernstein *et al*.[7] in a study conducted on male mice, which had been fed with a supplement of 0.20% DOC for 10 months. A similar result is described by Prasad *et al*.[13] who described changes like Peyer's hyperplasia plates in the small intestine of female mice. Adenomatous injury was also reported by Payne *et al*.[14] besides alterations of chronic colitis type, with no statistically significant differences between groups, in a study conducted for a period of eight months. These results were also found by Bernstein *et al*.[15].

Secondary bile acids in mice: intake dose in food vs. level excreted in feces

Like Bernstein, who reported DOC levels regrown in the mice feces fed for 90 days with a diet containing an additional of 0.20% DOC (more exactly, a 15 times higher level compared to the control group: 4.6 mg/g *vs.* 0.3 mg/g)[7], in our study: a slightly higher supplemented diet (0.25% DOC, corresponding to about

3 mg/kg of body weight amount ingested) determined a similar but lower increased level in the mice feces (11 times higher: 1.85 mg/g $-T_{DOC}$ *vs.* 0.16 mg/g -C, assessed after 140 days). A lower supplement (0.125% DOC mixed with LCA in same amount) decreased the excreted DOC amount (7 times higher: 1.16 mg/g $-T_{DOC/LCA}$ *vs.* 0.16 mg/g -C). The differences between the basic levels of DOC (recorded to the control groups used in the two mentioned studies: 0.3 mg/g[7] *vs.* 0.16 mg) may be due to the different time periods after which the amounts were recorded (90 days[7] *vs.* 140 days), but also due to the different conditions applied for the analysis of feces samples or due to the different species of mice used in experiment (B6.129PF2/J male[7] *vs*. C57BL6 female). Differences between the genders of mice were also reported by Zhang and Klaassen[16].

Again, increased LCA levels in the mice feces (also met in people, according to Reddy *et al*.[6] were reported in relation to the higher levels of secondary bile acids induced by the high fat diets. The results of this study show that the daily intake of 0.25% LCA in the food consumed on 140 consecutively days drive extremely up (233 times higher) the amount of LCA in the mice feces (4.66 mg/g $-T_{LCA}$ *vs.* 0.02 mg/g –C). If half of the LCA dose is replaced with DOC, the level of LCA in the feces gets comparable with that of excreted DOC (1.23 mg/g *vs.* 1.00 mg/g $-T_{DOC/LCA}$, subtracting C) and their combined amounts represents only a 13 times higher increase of these bile acids in feces (2.41 mg/g $-T_{DOC/LCA}$ *vs.* 0.18 mg/g –C). Therefore, the simultaneous ingestion and excretion of DOC and LCA can be consider as a particular situation regarding the levels reached by these bile acids in the feces.

With respect to the simultaneously presence of the two secondary bile acids (DOC and LCA) in the GI mice tracts (by exogenous intake), the experimental data show some unexpected levels of the amounts excreted into feces. Thus while to the control group, the normal excreted DOC value is approximately 10 times higher than that of LCA (group C, feces: 0.16 mg/g DOC *vs*. 0.02 mg/g LCA), the excreted values measured to the group $T_{DOC/LCA}$ (intake: 1.5 mg/kg DOC + 1.5 mg/kg LCA) are almost equal (feces: 1.16 mg/g DOC + 1.25 mg/g LCA). An intake of DOC in a double dose (group T_{DOC}, intake: 3 mg/kg DOC) totally inhibits the excretion of LCA in this mice group feces (group T_{DOC}, feces: 0.0 mg/g LCA), although an intake of LCA in same high amount (group T_{LCA}, intake: 3 mg/kg LCA) determined an excreted level of DOC slightly below the baseline level (feces: 0.12 mg/g DOC $-T_{LCA}$ *vs.* 0.16 mg/g DOC –C). These values suggest a possible interconnection of these secondary bile acids, probably by a mechanism of inhibition at certain concentrations, as well as changes in their endogenous regulating mechanism.

It has been reported out of the two secondary bile acids, LCA is the most toxic to colon cells and is approximately 20-fold more toxic toward colon cancer cells than deoxycholic acid[17].

The results of this study show that the amount of LCA in the mice feces (group T_{LCA}) is 233 times higher than the baseline level, so the exposure of the mice colon mucosa is much higher. This could be o possible explanation for the highest number of flat adenoma type lesions found in this group.

Progression to colon cancer proceeds by means of numerous changes in the colonic mucosa, progressing from normal tissue to "field defects" in the non-neoplastic flat mucosa, to hyperplastic polyps, to adenomatous polyps (adenomas), and, ultimately, to colon cancer[18].

It has been reported that colorectal flat adenomas have a greater tendency to develop into severe dysplasia and carcinoma than protruding adenomas[19].

It is a fact that lithocholic acid induces DNA damage and inhibits DNA repair enzymes[20]. These changes in DNA could be considered to be the first changes that appear in the molecular evolution of colon cancer. In this pathway a series of morphologically identifiable stages can be seen: initial localised colonic epithelial hyperplasia, followed by formation of adenomas that progressively enlarge and ultimately develop into invasive cancers i.e. the adenoma-carcinoma sequence[21].

This sequence can be viewed as a possible mechanism in the development of flat adenoma type lesions and cancerous lesions, a long term study being to validate this hypothesis.

Conclusions

Histopathological examination of the resected mice digestive tracts indicates that a daily ingestion of about 3 mg/kg LCA leads to the highest number of flat adenoma lesions (8) hyperplasia of Peyer's patches (25) and chronic colitis (2), compared to the ingestion of DOC or of both acids mixture (1:1), in the same amount. Neither rectal nor distant lesions were found, as well as precancerous or cancerous pathological lesions (that establish a causal relationship between bile acids and colon cancer) could not be identified. Instead, adenomatous colonic injuries occurred in a shorter period of time (six months), compared to the literature reported data.

In order to obtain more conclusive data, it is considered necessary to conduct the study over a larger period of time (12 months), to apply an immunohistochemical method and to correlate the data with human histopathological lesions.

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