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Role of Kefir Milk on The Pathogenesis of *Entamoeba histolytica*

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Abstract:

Kefir is fermented milk made from kefir grains consist mainly of lactic acid bacteria and yeast, it has several health-promoting properties, such as antimicrobial ,antitumoral, and immunomodulating effects. However, there are few scientific reports about the effects of kefir on parasites. This work, studied the biological activity of kefir fermented against *Entamoeba histolytica* parasite in mice at concentration (100% and 50%) compared with the metronidazole (flagyl) drug at concentration (30 mg\ml). The results showed that infected mice treated with kefir(100% and 50%) complete the eradication of parasite after (6^{th} and 8^{th} day) respectively post inoculation while in metronidazole treated group the complete the eradication occur after (8^{th} day) compared with control group which maintain shedding parasite until the end of experiment. The percentage of reduction parasite of treated groups were : kefir 100%was (81.1%) , kefir 50% was (76.4%) and for metronidazole was(75.1%). Histopathological it was study found that the parasite and metronidazole cause mucosal damage and inflammation while kefir reparation of the parasite damage in addition ,notice an increase in INF-γ levels was noticed in serum mice treated with both concentration of kefir and metronidazole reaching (428.8 , 419.8and 442) pg \ ml respectively compared with a range of positive and negative control, reaching (386.2, 233.2) pg \ ml. It also caused an increase of the concentration of secretory IgA in the intestinal tissue as it was in kefir groups and metronidazole (5.31 ,4.76 and4.14) ng \ ml respectively , compared with the positive and negative control groups, reaching (2.91 and 0.72) ng \ ml respectively. These results indicated that kefir could help in reducing the effect of *Entamoeba histolytica* or treating the parasite in patients especialy in immune suppressed patients .

Keywords: Kefir, *Entamoeba histolytica* , probiotics, INF-γ, sIgA.

دور لبن الكيفر(الفطر الهندي) على أمراضية طفيلي الأميبا الحالة للنسيج

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الخلاصة

يعد الكيفر حليب متخمر يصنع من حبيبات الكيفر، يتكون بصورة رئيسية من بكتريا حامض اللاكتيك وخمائر، ويمتلك العديد من الخصائص المعززة للصحة مثل مضاد للمكروبات ومضاد للسرطان ويؤثر على الجهاز المناعي. هنالك عدد قليل من البحوث العلمية حول أثار الكيفر على الطفيليات. تضمنت الدراسة الحالية اختبار الفعالية البايولوجية لحبيبات الكيفر ضد طفيلي الأميبا الحالة للنسيج *histolytica Entameba* في الفئران وبتركيزين(50% and 100%) وبالمقارنة مع عقار الميترونيدازول (الفلاجيل) وبتركيز (30 mg\ml). أظهرت النتائج أن الفئران المصابة والمعالجة بالكيفر قد توقفت عن طرح الطفيلي نهائيا في اليوم (6^{th}و8^{th}) على التوالي بعد التجريع، بينما أختفى الطفيلي قي براز الفئران المعالجة بالمترونيدازول تماما بعد اليوم (8^{th}) مقارنة مع مجموعة السيطرة والتي أستمرت بطرح الطفيلي حتى نهاية

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التجربة. وكانت النسبة المئوية لتناقص الطفيلي للمجاميع المعالجة كالأتي : كيفر 100% (81.1%)، كيفر 50% (76.4%)،المترونيداول (30 mg\ml) (75.1%). كما أظهرت دراسة الأمراضية النسيجية أن طفيلي الأميبا الحالة والميترونيدازول قد تسببوا بحدوث التهاب وتنخر لخلايا الامعاء بينما ساهم الكيفر في أعادة أصلاح نسيج الامعاء المتضررمن الطفيلي .كما لوحظ حدوث زيادة في مستوى INF–γ في مصل الفئران المعالجة بالكيفر(كلاالتركيزين) والمترونيدازول اذ بلغت نسبته (442, 428.8 , 419.8) pg\ml على التوالي مقارنة مع مجموعتي السيطرة الموجبة والسالبة أذ بلغت (233.2 386.2) pg\mlعلى التوالي. بالإضافة الى ذلك كانت هناك بزيادة تركيز IgA الافرازي في نسيج الأمعاء أذ كان تركيزه في مجموعتي الكيفر والمترونيدازول (5.31, 4.76, 4.14)ng\ml على التوالي، مقارنة مع مجموعتي السيطرة الموجبة والسالبة أذ بلغت (0.72و 2.91) ng\ml على التوالي. تشير هذه النتائج إلى أن الكفير يمكن أن يساعد في الحد من تأثير طفيلي أميبا الحالة للنسج أوفي علاج الطفيلي وخاصة في المرضى الذين يعانون من التثبيط المناعي .

Introduction

Amoebiasis isa human disease caused by many protozoal parasite like *Entamoeba histolytica* with or without clinical symptoms [1,2].Infection is occurred by ingestion of cyst from fecal contaminated water ,food or by food handlers [3].Amoebiasis is presently one of the three most common causes of death from parasitc disease, the world Health Organization founded that *E. histolytica* causes nearly 50 million cases and 100,000 deaths annually [4,5].

The cyst is resistant many environmental conditions like chlorine used in water and gastric acid [6].Symptoms may include bloody diarrhea, mild diarrhea ,tissue death, abdominal pain and peritonitis[7].Many drugs are used for the treatment of amoebiasis, most of them use is metronidazole [8], but many reported side effects like nausea, metallic taste and gastrointestinal disorders[9].

Propbiotics are defined as live food supplement microorganisms that confer health benefits when administered in enough amount [10].Kefir is a fermented milk produced by the effect of bacteria and yeast contained in kefir grain [11].Kefir grain are combination of lactic acid bacteria and yeast in matrix of sugar, lipids and proteins [12].It is at first very small but increase in size during fermentation [13]. These grains consist of complex group of lactic acid bacteria like (*Lactobacillus casi ,L. hilgardi, L.kefir, L. plantarum, L. delbrueckii spp* and *streptococcus lactis*),yeast (*Sacharomyces cerevisae* ,*Torulospora pretoriensis* ,*Candida lambica* and *Candida valida*) [14].Kefir consider as a natural probiotic [15].Regular kefir exhaustion can be help to relieve all intestinal disorders, reduce flatulence and create a healthier digestive system [16].Which provides beneficial bacteria and yeast, vitamins, minerals and protein, it has been used to help patients suffering from AIDS, chronic fatigue ,herpes and cancer ,antibacterial[17].Plant extracts, bee-derived products and probiotics are safety and inexpensive which use to treat intestinal parasite[18].

There are few scientific reports on the effectiveness of probiotics or fermented products as treatment for *E. histolytica* .This study investagate the ability of kefir-fermented milk in treated and protect mice from *E. histolytica* infection and high light on some of the changes that occur infection and treatment.

Material and Methods

Samples Collection:

Stool samples were collected from patients with diarrhea from privet laboratory randomly. Small amount of sample was examined on direct microscopic examination of feces to ensure that contain the parasite.

Culturing the parasite:

Small amount of positive stool sample was cultured on the LES-media(NIH modification of Boeock and Drboh lav's media) [19].Culture tube incubated vertically at $37C^0$ for 48h.For experimental inoculation, actively growing trophozoites were sediment after chilling the culture tubes for 5min in an ice-water bath ,and were finally suspended in PBS to final concentration of 1×10^6 trophozoite \ml .

Kefir preparation:

Commercial kefir grains from America\Losangeles were used to obtain the fermented milk [20].The granules were washed with water and incubated in milk in a proportion of 10% w\v for 24h

at 20 C^0.The product obtained was filtered to separate the fermented milk from the granules, which were washed again and seeded in fresh milk .Fresh kefir was prepared every 24h and was administered to mice absolute (100%) and diluted (50 %)in distilled water. The final concentration of bacteria and yeast was approximately 10^{12},10^5 for absolute and 10^6,10^2 c.f.u ml^{-1}respectively.

Animals:

Thirty six male albino mice aged 6-8 week, weighing 20-25gm were obtained from National Control Center for Drugs and Researches, the mice were housed under standard conditions and were fed with a conventional diet and water *ad libitum*, stool of them was examined before beginning of the experiment to make sure that the mice are free from any intestinal parasites.

Experimental design:

Experimental animals were immunosuppressed by hydrocortisone acetate (25 mg\ml) in a daily intramuscular dose of (0.1ml\mouse) for 5 days. 24 mice were inoculated with (0.1ml) contain (1×10^6 trophozoite), after (48hr) all mice feces were examined to confirm the infection occur, then the infected mice divided to (4groups) each group contain (6) mice, the remaining non infected mice kept as a negative control group. Then each group was inoculated as follow:

-CP(positive control):was only infected, given orally (0.1 ml) of normal saline.

-KA(kefir absolute): was given (3ml)of kefir (100%).

-KD(kefir diluted): was given (3ml) of kefir (50%).

-GM (group metronidazole) : was given(0.1ml**)**of metronedazole (30 mg\ml).

-CN(negative control): was given only (0.1ml) of normal saline.

Enumeration of the parasite:

Parasites in feces were enumerated according Magda *et al.* [21].Counting of shed parasite in (10) microscopic fields, calculation of the mean parasite count and the percent reduction in each group was determined.

Sufficient treatment calculation:

Sufficient treatments for kefir and metronidazole were measured according to the method of Xiao *et al* [22].

Quantification of mouse INF-γ in mice serum:

At the end of the Kefir and metronidazole treatment period, blood of the mice was with drawn from all groups and were subjected for separation of sera .INF-γ Concentrations were determined by commercially available ELIS Kit of mouse INF-γ(cat No.K0331138) Komabiotech.

Quantification of secretory IgA (sIgA) :

At the 7 th day post-infection and treatment ,four mice from each group were sacrificed and removed (1gm)from large intestine ,the concentration of sIgA were determined by commercially available ELIS Kit of Mouse Secretory Immunoglobulin A (sIgA) ELISA Kit (Cat No. MBS269144) Mybiosource.com.

Histopathological study:

At the end of experimental period, hematoxylin and eosin stained large intestine sections from mice of all groups were examined microscopically for histopathological changes.

Statistical analysis:

Data were computerized and statistically analyzed using the arithmetic mean and standard deviation, T-test and chi -squire test.

Results and discussion:

The present study showed the effective impact of Kefir in gradually reducing the number of parasites *Entameba histolytica* in infected mice with. As shown in Table-1, the feces of mice became clear from the parasite completely for KA group at the 6^{th} day and at the 8^{th} for KD group post inoculation.

The metronidazole treated group(GM) also causes gradually reducing in number of parasite shedding and became zero at the 8^{th} day after treated ,this reducing in numbers of parasites was statistically significantly($p<0.001$),while the control positive group(CP)continued shedding of parasites to the end of the test. Also, the percentage of reduction in parasites shedding for treated groups were : (KA)group was (81.1%) in comparison to the(KD) group and (GM) group which was (76.4% and 75.1%) respectively . There are significant differences ($p<0.05$) between treated groups as which is showed in Table-2.

Probiotic can kill or inhibit both prokaryotic and eukaryotic pathogenesis in the intestine by induce many mechanism through active molecular secretion (e.g. hydrogen peroxide, free fatty acids ,bacterocin like lactacin , reuterin, anisin), and enhanced Immune response, Lactic acid can change the local intestinal pH which directly inhibits the growth of the acid sensitive organism [23]. Any microorganism must be first adhere to the epithelial cell of the intestine to invade host cell and replicating occur, probiotic bacteria may compete for adhesion site and occupy common receptors on the intestinal epithelial cell [24]. Recently from invitro culture systems and animals models established that probiotic bacteria can be used for therapeutic and control both intestinal parasite and few non gut infections spread among human and veterinary animals [25]. Also shown that the probiotic yeast named *Saccharomyces boulardii* when combined with antibiotics show protective effect of parasite *E histolytica* and reduce the of disease symptoms [26].

Oliviera – sequeira *et al*.[27] reported that the inoculation of available *Bifidobacterium animalis* can induce resistance against *Strongyloides venzuelensis* infection in mice.

Table 1- The number of *E.histolytica* parasites in Kefir absolute , kefir dilute ,metronidazole and control group.

	Group				
Day after treatment	**CP**	***KA**	***KD**	***CM**	
1	2.5± 0.57	3± 0.81	2.75± 0.95	2.5± 0.57	
2	6± 0.81	4.25± 0.95	3.25± 0.5	5± 0.81	
3	8± 0.81	5±0.81	5±0.81	6±0.81	
4	8± 1.82	3.25± 0.95	4.75± 0.95	4± 0.81	
5	9.5± 1.29	1.5±0.70	3± 0.81	2.5± 0.57	
6	10.75± 0.95	0	2± 0.81	2± 08.1	
7	11.75± 0.95		1±0	1± 0	
8	11.5±1.29		0	0	
9	12.5±1.29				
10	12± 0.81				
11	0				
* (P<0.001)					

Table 2- The percent reduction in *E.histolytica* parasites shedding among treated groups.

Mouse groups	Percent reduction in parasite shedding %
*Kefir A	81
*Kefir D	76.4
*GM	75.1
Stastical analysis	P<0.05

Serum level of mouse INF-γ:

As shown in Table-3 the concentration of INF-γ in all serum treated groups (KA, KD, CM) were increasing significantly (p<0.05) and became (442,428.8 and 419.8) pg\ml respectively in comparison to CP groups which was only (386.2) pg\ml, while the CN was (233.2) pg\ml.

Probiotic can modulate release many cytokines like (INF-γ,TNF-α,IL-12 and IL-10) which play a main role in maintaining the equilibrium between necessary and excessive defines mechanism [28]. Exogenous IFN-γ can activates neutrophils and macrophages for killing *E. histolytica in vitro* [29]

Seydel *et al*. [30] suggested that IFN-γ and nitric oxide (NO) are important in host defense against the protozoan parasite *E. histolytica* , also it able to activate host neutrophils and macrophages to kill amoebic trophozoites *in vitro* and may play similar function in the murine model of amebic liver abscess. Oral administration of kefir can induce both Th1 response by Th2 cytokines like (IL-2,IL-4, IL-12, IFN-γ) on the intestinal mucosal in mice [31].

Table 3- INF-γ level in sera of different treated groups

Mouse groups	INF-γlevel (pg\ml)±SD
*CN	233.2±1.64
*CP	386.2±3.03
*Kefir A	442±3.6
*kefirD	428.8±7.7
GM	419.8±2.86
Stastical analysis	P<0.05

Concentration of sIgA in intestinal tissue:

The concentration of sIgA was determined by using ELISA-kit. Table-4 shows that at 7thday post infection and treated significantly increasing (P<0.005) occur in sIgA concentration which was recorded in intestine (KA and KD)which became (5.31 and 4.76 ng\ml) respectively ,compared with GM group was which (4.142 ng\ml) , while the CP and CN groups was (2.91 and 0.72 ng\ml) respectively, suggesting that the sIgA level was induced by kefir.

Thoreux and Schmuker [32] found that fed kefir to young mice and old rats led to faster mucosal immune response also increase IgA-secreation, sIgA in the gut consider as non-specific defense mechanisms and intimate cooperation with innate immunity [31].

Many studies establish the ability of fermented milks to support humoral immunity by increasing secretion intestinal IgA [33, 34], sIgA is considered the first line of specific defense against natural infections in the vast area occupied by mucosal surfaces [35].

The sIgA purified from a pool of anti-protease-positive samples showed a strong inhibitory effect on the *E. histolytica* proteolytic activity *in vitro* [36].

Carrero *et al*.[37] Found that the secretory (IgA) anti-*E. histolytica* antibodies in the saliva of patients with intestinal amoebiasis an inhibit amebic adherence to a monolayer of MDCK (Madin-Darby canine kidney) cells.These results reflect the ability of secretory IgA antibodies to prevent of *E. histolytica* adherence to epithelial cells.Mucosal IgA antilectin antibody response is associated with immune protection against *E. histolytica* colonization [38].

The sIgA response developed against some antigens of *E. histolytica* trophozoites during the intestinal infection in humans and experimental rodents confers temporal protection against the re-infection, probably by inhibiting the adherence of the parasite to the colonic wall [39].

Cysteine proteinase activity is predominately responsible for the degradation of human IgA by *E. histolytica* [40]. *S.boulardii* can be treating giardiasis when combined with metronidazole therapy and can stimulation of IgA secretion [41].

Table 4- Secretion IgA concentration (ng\ml)in intestinal tissue of different treated groups and control group

Mouse groups	sIgA concentration (ng\ml))±SD
***CN**	0.72±0.11
***CP**	2.91±0.50
Kefir A	5.31± 0.83
***kefirD**	4.76±0.57
***GM**	4.14±0.35
Stastical analysis	P<0.05

Histopathological study:

Histopathological changes of the intestine after *E.histolytica* and after therapeutic with kefir and metronidazole was study as following:

-CN (control negative): showed normal structure as shown in Figure-1.

-CP (infected): showed hydropic degeneration and vacuolated epithelial cell due to necrosis and mild inflammation Figure-2.

-KA and KD: looks like normal structure as shown in Figure-3, only little increase occur in goblet cells no pathological changes were noticed.

-CM: showed inflammation, hydropic degeneration, mild increase in goblet cell, short length of villi Figure-4.

Mild irritation of the epithelium produced by soluble amebic products, Irritation stimulates goblet cells to release mucus, at the same time that it increases its production, thus explaining the glandular hyperplasia, also amebic toxins may produce edema of the underlying epithelium [42].

Teruya *et al*., [43] study the protective effect of Kefir against the gastrointestinal damage associated with radiation therapy. The histological and immunohistochemical examinations revealed that the diluted Kefir solutions protected the crypts from radiation, and promoted crypt regeneration.

Kefir is established of milk ,therefore it can buffer the pH of the stomach when ingested ,this provide time for many of the bacteria to pass through to the upper small intestine [44], Although yoghurt which well known indifferent countries than kefir consider an important probiotic products [45], it enhance proliferation and colonization rate of beneficial bacteria in the intestine which

conversely prevents growth of certain pathogens [46] and contain large number and different bacteria and yeast during fermentation. The yeast and bacteria produce variety of component that gives kefir singular taste, texture and proving to be bioactive. At least one exopolysaccharide, proteinase and large number of bioactive peptides has been found in kefir, thus kefir consumption not only affects digestion, but also effect on metabolism and immune function in human.

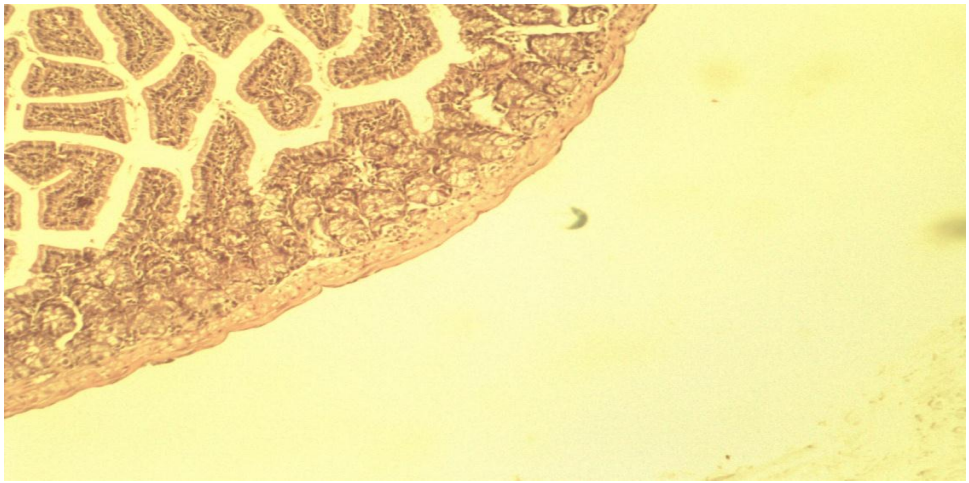

Figure 1- Section of large intestine from mice of negative control showed normal structure (H&E; 200X).

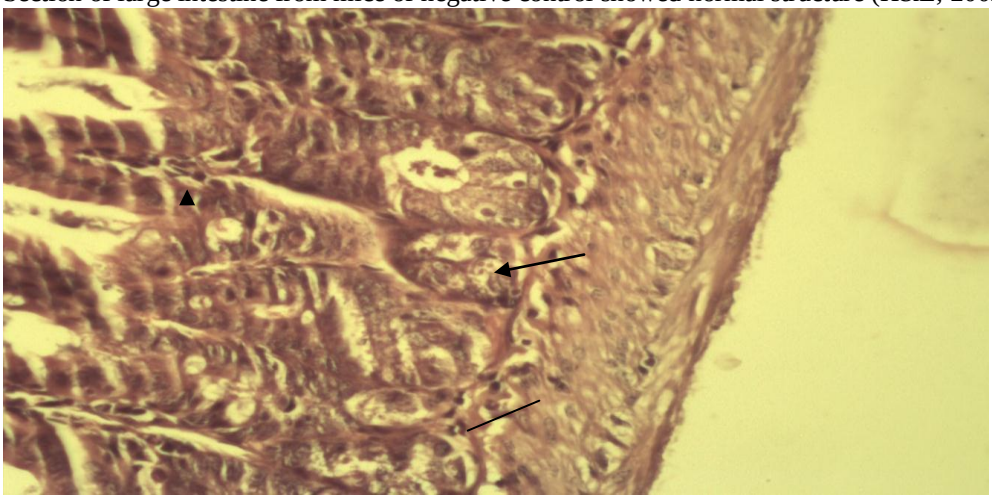

Figure 2- Histopathological section of large intestine from mice of positive control showed necrosis (⟶) and mild inflammation (——) . (H&E), 400X

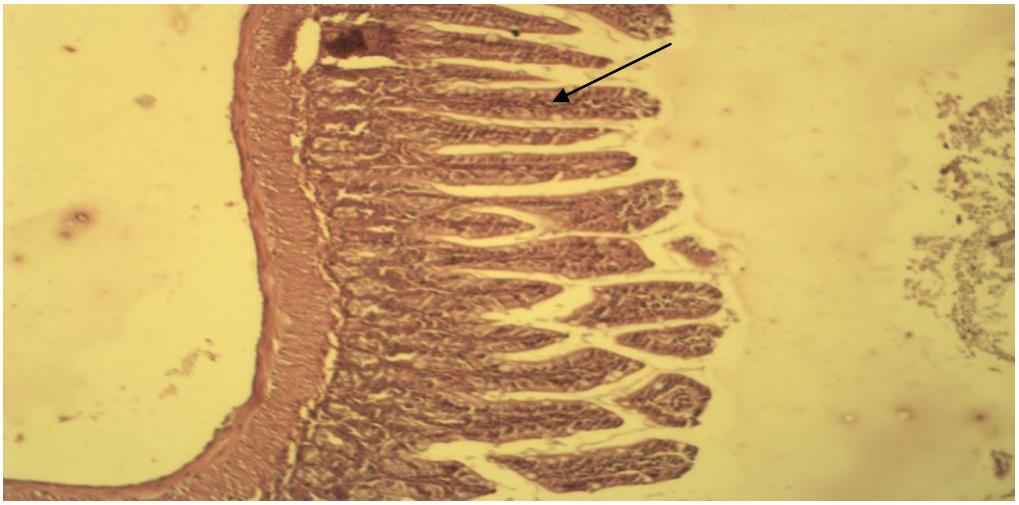

Figure 3- Section of large intestine in mice treated with Kefir, showing near normal appearance of colonic mucosa mild increase in goblet cells. (H&E), 200x

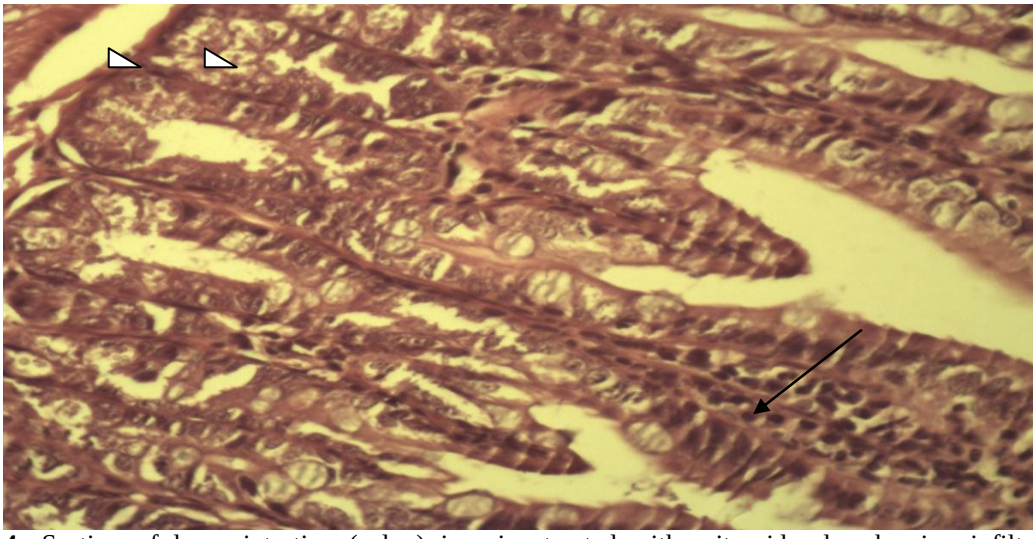

Figure 4- Section of large intestine (colon) in mice treated with mitronidazole ,showing infiltration of lymphocytes (⟶) , necrosis (◺) and short length of villi . (H&E) , 400x.

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