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Original Article

Folate acid analysis in predicting complicated appendicitis in animal model *oryctolagus* cuniculus

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ABSTRACT

Background: Appendicitis usually presents as an acute disease presentation within 24 hours. Folic acid plays a role in the synthesis of DNA nucleotides, methionine amino acids, and homocysteine (Hcy) level regulation, also regulates DNA stability through DNA methylation, synthesis, and repair mechanisms. This study investigated whether reduction of folic acid level affected the risk of inflammation.

Material and Methods: This study was designed as an experimental analytic study that involved twenty male rabbits weighing for 2.500-3000 g. The appendix lumen was then obstructed with silk no.O. at 18, 24, 36, and 48-hours. Blood samples were taken for serological examination of folic acid using ELISA method. Appendectomy was performed for histopathological examination.

Results: There were significant differences in folic acid levels between 18-hours and 24-hours group, 24-hours and 36-hours group, 24-hours and 48-hours after the intervention done.

Conclusion: Decreased levels of folic acid could be a predictive factor for complicated appendicitis in experimental animals.

Keywords: folic acid, predictive factor, complicated appendicitis

Introduction:

Appendicitis is a spectrum of diseases ranging from simple inflammation to perforation with extensive contamination. The main etiology of this disease is an appendiceal lumen obstruction caused by various factors, including lymphoid hyperplasia, appendicolith, foreign bodies, parasites, or malignancy. Obstruction of the appendiceal lumen causes appendix distension because appendix continuously secretes mucus, and then bacteria can multiply.[1]

In children, appendicitis mostly requires an immediate surgery; the reason why most children are hospitalized. In USA, there were 60.000-80.000 cases of appendicitis in children every year; it is estimated that the risk of appendicitis in boy and girl were 9% and 7% respectively. Despite having a lower incidence rate, girls likely to have a higher rate of appendectomy (23.1%) than boys (12%).[2,3] Appendicitis usually presents an acute disease presentation within 24 hours, but sometimes appears as chronic condition. The course of symptoms varies, but usually develops from the beginning of appendicitis (12-24 hours), and the risk of perforation is increasing (>48 hours). Seventy-five percent of patients

present after the onset of symptoms within 24 hours. While the risk of appendicular rupture varies, 2% of cases occurred at 36 hours, increased about 5% every 12 hours.[4,5]

Consistently, lots of literatures explained that appendicitis in patients who experienced symptoms in long duration were often associated with the burden of perforated appendicitis, in both pediatric and adult. The time span of perforation risk is considered as symptoms presented in 24 hours, 36 hours, or more than 48 hours. In cases of symptoms over 36 hours, risk of perforation significantly increases, even progresses within 12-hours (48-hours). It is also stated that there was a tendency of perforation as symptoms of appendicitis went longer.[6]

This study consisted of four sample groups and after animal model intervened, the blood samples were serially taken at 18-hours, 24-hours, 36-hours, and 48-hours from the heart of rabbits and appendectomies were performed for histological examination. Meanwhile, Katz's study demonstrated that they were also done at 0-hours, 6-hours and 12-hours. A study that conducted by Erjan Fikri in 2019, blood and tissue histological samples were taken 13 hours after all animal models intervened. [7,8]

Based on previous literatures, the authors decided to set measurement time in acute phase (18 hours); Erjan Fikri set the time was at 13 hours after being intervened and Howel *et al*. stated that acute appendicitis might occur at 24 hours. This is why authors set median time between these two. Meanwhile, the authors took measurements of the risk of complicated appendicitis that might occur at 24-hours, 36-hours, or more than 48-hours in the setting of complicated appendicitis. [4,6]

Establishing diagnosis of appendicitis needs several modalities, one of which is the *pediatric appendicitis score* (PAS). A PAS score of 4-6 will require a repeat imaging, but close observation is still needed to prevent complicated appendicitis; it might use ultrasound, though it is operator-dependent.[9]

Moreover, diagnosis of appendicitis needs to be supported by other investigations, such as laboratory and radiological examinations. There is no significant development of diagnosis in child with appendicitis. This condition is based on communication and inspection difficulties.[10,11]

Materials and Methods:

This study was an experiment-analytical study to investigate levels of folic acid as a predictor of complicated appendicitis, as shown in figure 1. The animals model that used in this study were healthy male rabbits (*Oryctolagus cuniculus*), weighting from 2.500-3.000 g. Though smaller, they have similarities in physiology with humans. The sample size was determined based on Federer formula; amount of sample for each group were 5 male rabbits. The inclusion criteria were male rabbits weighting from 2.500-3.000 g. Rabbits that have abnormalities in the digestive tract, especially on appendix diagnosed at the time of laparotomy were eliminated from this study.

All samples were twenty male rabbits (*Oryctolagus cuniculus*) weighting from 2.500-3.000 g which been given intramuscularly with 0.5 mg/kg BB *Ketalar* lead no effect on spontaneous breathing. Then, abdominal exploration was carried out with a midline incision and the appendix was identified. Silk no.O was inserted at the base of appendix through the mesentery, and the lumen of appendix was being obstructed by binding the silk no.0 in all rabbits. The abdomen was closed. Finally, they are being returned into cages.

After the appendix was obstructed, the sample had undergone 4 predetermined time: 18-hours, 24-hours, 36-hours, and 48-hours. Folic acid levels were also examined. Following that, the rabbit was once undergoing appendectomy, the blood sample was taken to measure folic acid levels. The rabbits were terminated after being intervened.[8]

On histological examination, all appendices were drown in *4% formaldehyde*

and fixed with paraffin blocks. Specimens were stained with hematoxylin-eosin and examined using a microscope.

Flowchart of Study Method

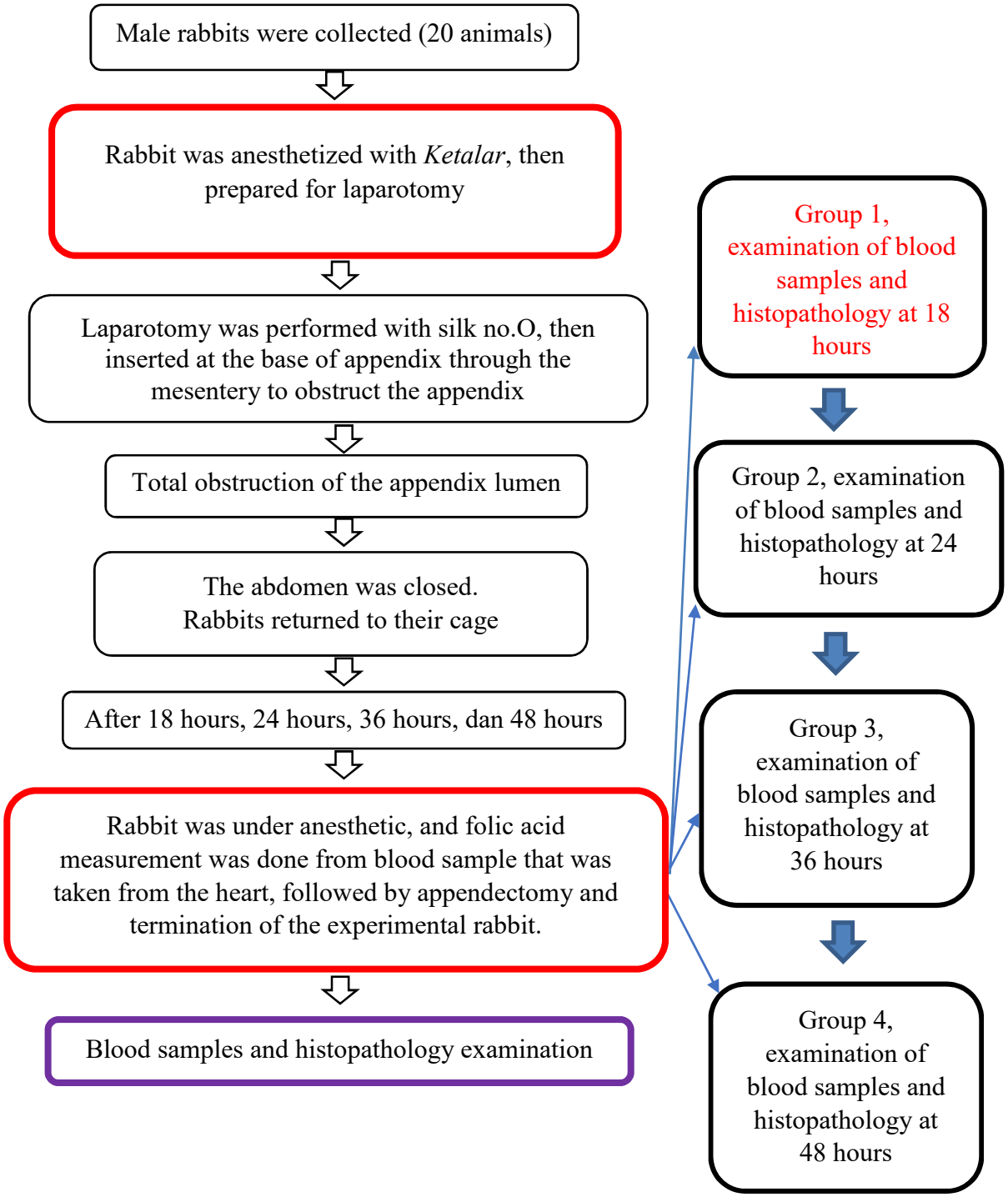

Figure 1. Flowchart

Statistical Analysis:
Data was checked for the completeness, coding, tabulating data, and data entry. Analysis of research data are presented in mean±SD for normally distributed data and median (min-max) for data that was not normally distributed. To analyze difference between these two groups, *T-dependent test* will be tested if data was normally distributed or *Man-Whitney test* when the data was not normally distributed. To analyze differences in more than two groups, ANOVA tests will be tested if data was normally distributed or *Kruskal Wallis test* if data was not normally distributed.

The significant limitation is considered as $p<0.05$ which means there is significant relationship between these two variables. Data analysis was performed using statistical software.

Result:
Distribution of Samples Based on Histopathological
The distribution of histopalogical results were examined with pathology examination, then classified based on operational definitions into two general classifications: acute appendicitis and complicated appendicitis (including phlegmonous appendicitis, gangrenous appendicitis, and perforated appendicitis) shown in table 1.

Table 1. Distribution Based on Type of Appendicitis.

Measurement Time	Type of Appendicitis	Total (%)
18	Acute Appendicitis	4 (80%)
	Phlegmonous Appendicitis	-
	Gangrenous Appendicitis	-
	Perforated Appendicitis	1 (20%)
24	Acute Appendicitis	2 (40%)
	Phlegmonous Appendicitis	1 (20%)
	Gangrenous Appendicitis	1 (20%)
	Perforated Appendicitis	1 (20%)
36	Acute Appendicitis	1 (20%)
	Phlegmonous Appendicitis	1 (20%)
	Gangrenous Appendicitis	1 (20%)
	Perforated Appendicitis	2 (40%)
48	Acute Appendicitis	1 (20%)
	Phlegmonous Appendicitis	1 (20%)
	Gangrenous Appendicitis	2 (40%)
	Perforated Appendicitis	1 (20%)

Histopathological distributions were shown in figure 2, figure 3, figure 4, figure 5, figure 6, and figure 7. Vast majority of acute appendicitis was dominated at 18-hours post-intervention, meanwhile in complicated appendicitis, it was found to occur frequently at 36-hours and 48-hours post-intervention. While the total distribution of acute appendicitis were 8 samples (40%) and those with complicated appendicitis were 12 (60%) samples of 20 animals in table 2.

Table 2. Distribution of Appendix Frequency in Animal Model's Histopathology

Type of Appendicitis	Mean ± SD	Median (Min-Max)
Acute Appendicitis	101,73 ± 11,64	104,93 (86,19-116,14)
Complicated Appendicitis	99,60 ± 20,56	102,06 (54,31-122,24)

Figure 2. Gross pathology image of sample's Acute Appendicitis to complicated appendicitis based on measurement time at 18 hours, 24 hours, 36 hours, and 48 hours

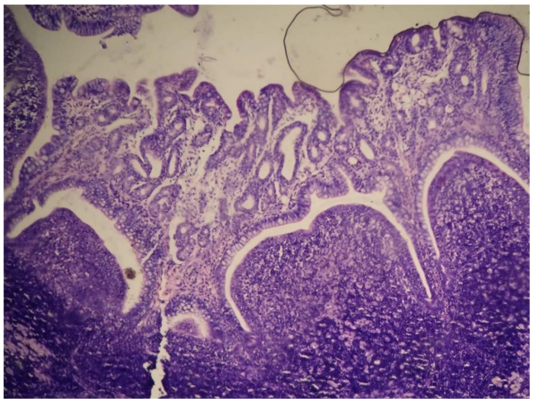

Figure 3. Histology image of Normal Appendix

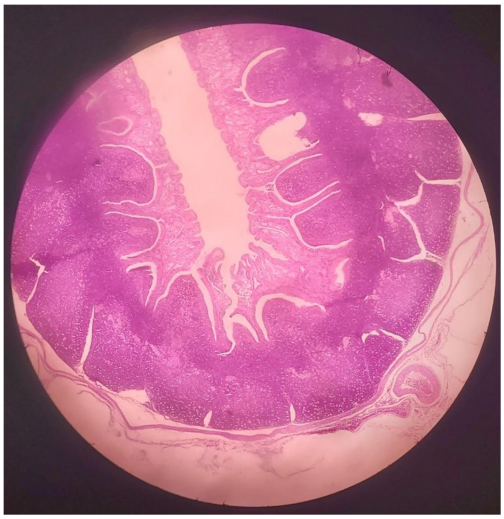

Figure 4. Histopathology image of acute appendicitis in rabbits. Severe infiltration of polymorphonuclear inflammatory cells

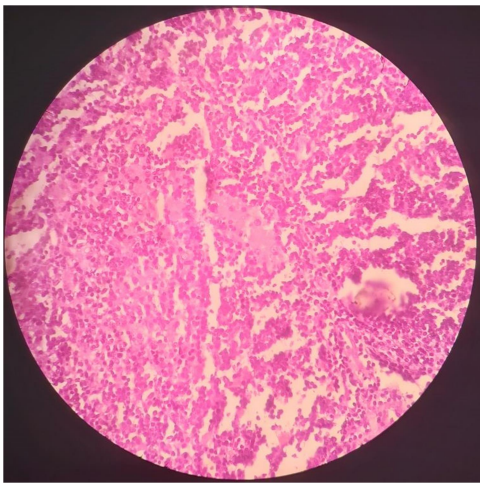

Figure 5. Histopathology image of phlegmonous appendicitis in rabbits. Neutrophil infiltration in muscularis propia

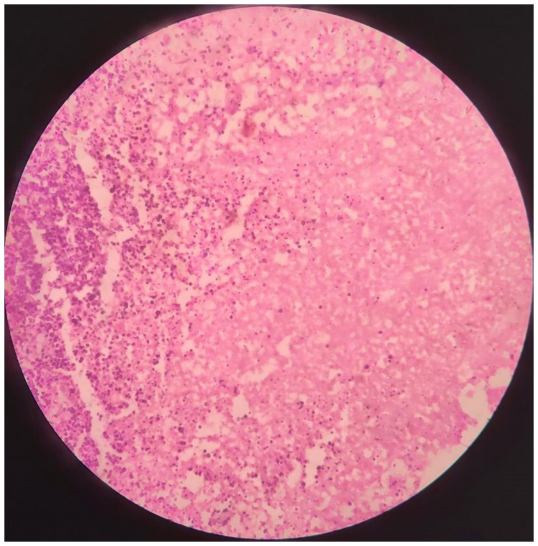

Figure 6. Histopathology image of gangrenous appendicitis in rabbits. There is necrosis in the mucosal epithelial layer and gangrene in the appendix

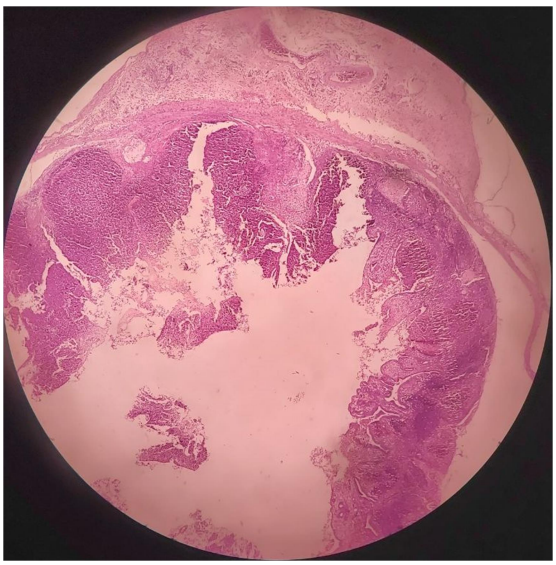

Figure 7. Histopathology image of perforated appendicitis in rabbits. There is damage in all layers of the appendix, with visible fat into the appendix layer.

Comparison of Folic Acid Levels in Animals Model Based on Measurement Time

Analysis with enzyme-linked immunosorbent assay (ELISA) obtained data of folic acid levels for all groups (18-hours, 24-hours, 36-hours, and 48-hours) which were normally distributed (p>0.05). That's why Anova test was conducted for looking at the difference in folic acid levels between groups. The results showed that there were differences

in folic acid levels between groups with $p<0.05$ in table 3.

Table 3. Differences levels of folic acid animals model based on time of measurement

Measurement Time	Mean ± SD	Median (Min-Max)	*p value**
18 hours	104,70 ± 9,65	107,47 (89,57 - 115,45)	0,044
24 hours	79,07 ± 18,93	79,07 (54,31 - 98,56)	
36 hours	109,10 ± 14,96	115,56 (86,19 - 122,24)	
48 hours	103,97 ± 16,40	108,43 (85,95 -121,74)	

* Anova Test; *p value* <0.05 was considered statistically significant

Comparison of Post Hoc Test Results toward Folic Acid Levels

Furthermore, the post hoc test was conducted using *Least Square Difference* (LSD) to see the difference of folic acid levels in the groups. The results showed a significant difference in levels of folic acid between groups of 18 hours with 24 hours, 24 hours with 36 hours. and 48 hours. There were no significant differences between the 18 hours with 48 hours groups and between 36 hours with 48 hours in table 4.

Table 4. Post Hoc Test Results toward Folic Acid Levels

Measurement Time		*p values*
18 hours	24 hours	0,023*
	36 hours	0,652
	48 hours	0,940
24 hours	36 hours	0,010*
	48 hours	0,027*
36 hours	48 hours	0,599

*Significant with Post Hoc LSD

Discussion:

Folic acid is a B vitamin that comes from a natural sources, such as green leafy vegetables or folic acid-enrich food supplementation. The role of folic acid on the human body depends on reduction of folic acid compounds, such as tetrahydrofolates (THF) that caused reduction in one unit of carbon (1C), therefore this reaction affects the synthesis of DNA nucleotides, amino acid methionine, and regulation of homocysteine (Hcy) levels.[13]

The relationship between chronic inflammatory conditions, such as inflammatory bowel disease and carcinoma have a strong correlation, and possibly involving folic acid role in regulating DNA stability through DNA methylation, synthesis, and repair mechanism.[14] According to Jones et al., study of "Folate and Inflammation - links between folate and features of inflammatory conditions" showed a relationship between folic acid level with an increased risk of inflammatory diseases. Low folic acid serum and elevated levels of homocysteine (Hcy) are usually reported in patients with chronic inflammatory disorders, such as inflammatory bowel disease and rheumatoid arthritis.[15]

Folic acid has been extensively studied in cardiovascular disease and cancer, with an increased risk of this disease in those with inadequate levels of folic acid. Folic acid is also a contributing factor to inflammation, other studies have shown relationship between folic acid status with cardiovascular disease and chronic inflammatory diseases, such as endothelial dysfunction. Endothelial dysfunction is defined as a decrease in vasomotor tone regulation through unbalanced vasodilator and vasoconstrictor levels, which causes the endothelium to shift towards a proinflammatory state. Folic acid can prevent endothelium dysfunction by maintaining levels of homocysteine (Hcy), vasodilators, and nitric oxide (NO).[16,17]

In a randomized clinical trial of another study, it was explained that giving folic acid supplementation with 0.4 mg or 0.8 mg dose daily for 8 weeks resulted significant reduction of DNA damage. For comparison, in vitro and in vivo studies have showed that folic acid deficiency will cause DNA damage by reducing thymine, which causes uracil to

become disrupted in DNA and inhibit DNA repair. This study also explained that folic acid dosing level also determine the effect on the risk of inflammation.[12]

In this study, 8 rabbits (40%) had acute appendicitis, 3 rabbits (15%) had phlegmonous appendicitis, 4 rabbits (20%) had gangrenous appendicitis, and 5 rabbits (25%) had perforated appendicitis. This showed several variations of appendicitis that occurred in this study. Also, this study demonstrated that data of folic acid levels for all groups (18-hours, 24-hours, 36-hours and 48-hours) were normally distributed (p>0.05), so Anova test conducted to see difference of folic acid levels between groups. It showed that there was difference in folic acid levels between groups (p<0.05). Furthermore, post hoc test was conducted to see the difference of folic acid levels within groups using *Least Square Difference* (LSD). The results showed significant difference of folic acid levels between groups of 18-hours with 24-hours, 24-hours with 36-hours and 48-hours.

It is consistent with previous studies which mentioned trends of lower folic acid levels, more likely to experience complicated appendicitis.[16]

Folic acid shows an association between folic acid levels and inflammatory diseases.[9]

Folic acid improves oxidative stress in the gastric mucosa. This results inhibition of acid output, an important factor in the pathogenesis of gastric ulceration. Folic acid can prevent endothelium dysfunction by maintaining levels of homocysteine (Hcy), vasodilators, and nitric oxide (NO).[19]

Giving folic acid supplementation of 0.4 mg or 0.8 mg dose daily for 8 weeks leads significant reduction of DNA damage.[20]

Low folic acid status also has a strong correlation in increasing the risk of carcinoma, in case folic acid regulating DNA stability through DNA methylation, synthesis, and repair mechanism.[12]

Folic acid provides an evidence of the potential anti-oxidant and anti-inflammatory effects of melatonin and folic acid. And lower folic acid levels in patients with ulcerative colitis when compared with the control group. This difference was considered statistically significant (p <0.001).[18]

Conclusion:

The result of this study demonstrated that folic acid levels for all groups (18-hours, 24-hours, 36-hours, and 48-hours) were normally distributed (p>0.05), so that Anova test was conducted to see difference of folic acid levels between groups. It also showed that there was difference in folic acid levels between groups (p<0.05). Furthermore, post hoc test was conducted to see the difference of folic acid levels in group, using *Least Square Difference* (LSD) showed significant difference of folic acid levels between groups of 18 hours with 24 hours, 24 hours with 36 hours, and 48 hours.

Finally, it was concluded that decrease of folic acid levels could be a significant predictive factor for the burden of complicated appendicitis in animal model.

Recommendation:

Study approval was obtained from the animal research ethics commission of the Biology Laboratory, Faculty of Mathematics and Natural Sciences, University of Sumatera Utara.

Study Limitation:

This study had only few samples, thereby lacks of samples to be generalized in community. Further studies needed to evaluate and support the result of this study. Also, not all medical facilities provide laboratory work-up of folic acid serum so it must be limited to investigate folic acid level as predictive factor of acute appendicitis.

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