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Effectiveness of Growol to Prevent Diarrhea Infected by Enteropathogenic *Escherichia coli*

K.D. Prasetia[1], T.W. Kesetyaningsih[2]*

[1]Program Study of Nursing, Faculty of Medicine and Health Sciences, Muhammadiyah University of Yogyakarta
[2]Department of Parasitology, Faculty of Medicine and Health Sciences, Muhammadiyah University of Yogyakarta

Abstract: Diarrheal disease is one of 10 leading causes of hospitalization in Indonesia in 2010. Prevention of diarrhea by maintaining personal and environment hygiene often do not go well in the community, especially in children. Researches on probiotics indicate that probiotics were able to protect the gut from pathogenic infection. Growol is a traditional food made from fermented cassava and containing probiotics. This study aims to prove the effectiveness of growol to prevent diarrhea due to EPEC (*E. coli* enteropathogen) in mice. The experiment was using *Rattus norvegicus* as animal model, with criteria male, aged 2-3 months and weigh 150-200 grams. Twenty four rats were divided into 4 groups: negative control (no treatment), positive control (cotrimoxazole), treatment growol 100% and 50%. Growol given from 7 days before infection until the end of the study, whereas the treatment was given on day 2 after infection for 4 days with a dose of 17.28 mg/mouse/day. Infectious dose is 1 ml 106 cfu/ml. The observation was conducted every day for 4 days starting at day 2 after infection. Dependent variable was the frequency of defecation, stool consistency and body weight. The results showed that administration of growol 100% and 50% effective in preventing diarrhea. It shown by the significant differences in frequency of bowel movement and stool consistency compared to negative and positive controls ($P < 0.05$). Giving growol 100% seems more potential than 50% but lose weight, meanwhile growol 50% increase body weight even though after the mice were infected.
Keywords: antidiarrhea, EPEC, growol, probiotic.

Introduction

Diarrhea and pneumonia are 2 major caused for 40% of children death around the world in every year[1]. Diarrhea is still be a health problem with mortality rate of 1.74% -2.94% (2000-2010) in some districts in Indonesia which are experiencing extraordinary events (KLB)[2]. Rotavirus and *E. coli* are the most common cause of diarrhea in developing countries[3]. Enteropathogenic *E. coli* (EPEC) is a strain of *E. coli* that cause diarrhea. Unscientific treatment of diarrhea was reach 60% as the use of antidiarrheal drugs, antibiotics and traditional medicines[4]. Growol is Kulon Progo (DIY) native food which made from fermentated cassava[5]. *Lactobacillus plantarum* and *Lactobacillus rhamnosus* are dominant bacteria which get involved in growol production[6]. *Lactobacillus* and *Bifidobacteria* were has been proved has some benefits to health[7]. This study aims to reveal the effectiveness of growol as diarrhea prevention in rats (*Rattus norvegicus*) model which infected with EPEC. We expect that the results of the research can provide information of growol benefits against bacterial infection in intestine.

Methods

Experiment was carried out in 24 male rats, aged 2-3 months, weighing 150-200 gram which divided into four groups, consisting 6 rats in each group. The groups were (1) negative control (without treatment) (2) positive control (sulfamethoxazole-trimethoprim), (3) growol 50% treatment and (4) growol 100% treatment.

Dosage

Growol was obtained from traditional market in Sentolo, Kulon Progo. Rats of growol 50% treatment group were fed with 10 grams of growol which mixed with 10 grams of rat food pellets, while rats of growol 100% treatment group were fed with 20 grams of growol. Food was given once a day. EPEC obtained from Health Laboratory of Yogyakarta as the starter. The EPEC bacteria then subcultured in nutrient broth medium for 24 hour at 37 ° C which performed in the Laboratory of Microbiology Medicine Faculty and Health Sciences of UMY. One ose of bacterial colonies suspended in 2 ml of BHI (brain heart infusion) until reach the standard Brown III turbidity (108 cfu / ml). EPEC infection dose was 106 cfu/ ml of saline solution[8]. Drugs were used for the positive control which is a combination of 400 mg Sulfamethoxazole and trimethoprim 80 mg. The dose which is given to rat models was 17.28 mg /rat/day. This dose was obtained from the calculation of the human dose (480 mg 2x/day) which converted to rats by conversion rate for mice 200 grams (0,018).

Treatment

Rats were acclimatized for 7 days in a cage trial before treatment then placed in individual cages. Growol was given orally for 13 days, started from 7 days before infected. EPEC infection conducted orally using feeding tube for 6 days starting on the 7th research day, Treatment with trimethoprim-sulfamethoxazole performed for 2 days on day 5 and 6 after the initial infection.

Diarrhea is defined as an abnormally frequent of bowel movement. There were some feces condition, feces was in normal condition when the feces is solid and there is no fluid. Feces categorized as mushy when it shaped and soft but not liquid. Semi-liquid stool is when the feces is shaped and contained fluid in the surrounding. Liquid stool stool is when the feces is not shaped and in the form of a liquid. Stool consistency was scored, from 0 to 3 (from the normal to the liquid). Based on preliminary tests, diarrhea was occur at 48 hours after infection. Frequency of defecation is the number of feces that found every day. The observation was begun at 48 hours after infection and performed twice a day for 4 days by counting the number of fecal matter which found in the base paper. As supporting data we also measured the changes of rat body weight during the study.

Analysis

Two way anova was used to measure the significance of differences among research groups and post hoc Tukey HSD was used to measure the significance of differences between each group with others.

Results

Frequency of defecation. Frequency of defecation is the number of defecation every day, for 4 days starting 48 hours after infection. The observations presented in Figure 1.

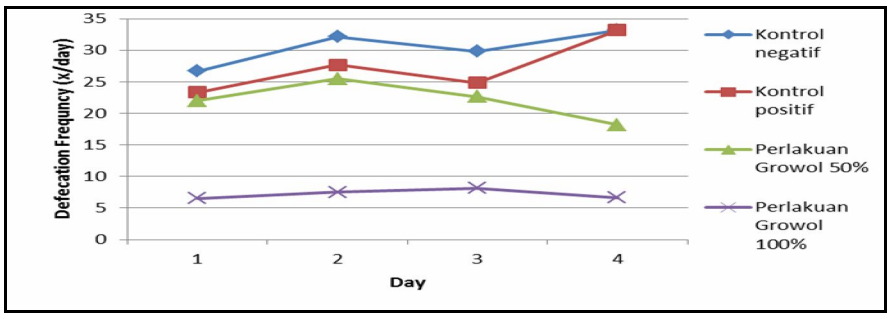

Figure 1. Average of defecation frequency (x/day) for 4 days start at 2 days post infection

Figure 1 shows the frequency of defecation dynamics the observation for 4 days. It shows that the average frequency of defecation in the negative control group (without treatment) is the highest 26.17 (SD ± 8.94) - 33.17 (SD ± 1.09) from day 1 to day 4. In both of growol 50% and 100% treatmet groups could reduce

the frequency of bowel movements which appear lower than the positive control with amount 6.50 (SD ± 5.70) on days 1 and relatively stable until day 4 with amount 6.67 (SD ± 5.34).

Normality Test shows normal distribution of the average frequency of defecation in control and treatment groups (p> 0.05), whereas the variation using Levene's test of homogeneity test shows p> 0.05 means that the same data variation as ANOVA test. Two Way ANOVA test showed significantly difference of frequency of defecation in each animal model groups (F count = 843 287; p = 0.00) which followed by Tukey HSD test (presented in Table 1).

Table 1. Frequency of defecation in each animal model groups (Tukey HSD test)

Group	P-value	Significancy
Negative Control	0.446	Not significant
NegControl-Treatment Gwol 100%	0.000	Significant
NegControl-Treatment Gwol 50%	0.010	Significant
PosControl-Treatment Gwol 100%	0.000	Significant
PosControl-Treatment Gwol 50%	0.137	Not significant

Tukey HSD test showed that the average frequency of defecation between negative control and positive control have no difference (p = 0.446). Negative control was significantly different (p<0.05) with both of 100% and 50 % growol treatment group. Positive control shown not significantly difference (p = 0.137) compared with 50% growol, but shown significantly different with the 100% growol (p = 0.000). This result suggests that growol administration for 7 days prior to EPEC infection effectively decreased the frequency of defecation, and giving 100% growol were more effective than antibiotics Sulfamethoxasole-trimethoprim.

Stool Consistency. Stool consistency quantified by giving the score after observation of stool. The score 3 is liquid, 2 is semi-liquid, 1 is flaccid, and 0 is solid. The results of the average stool consistency in all groups for 4 days observation presented in Figure 2. Two way ANOVA test results showed that there is significantly difference in the average scores of stool consistency in each animal model group (p = 0.017).

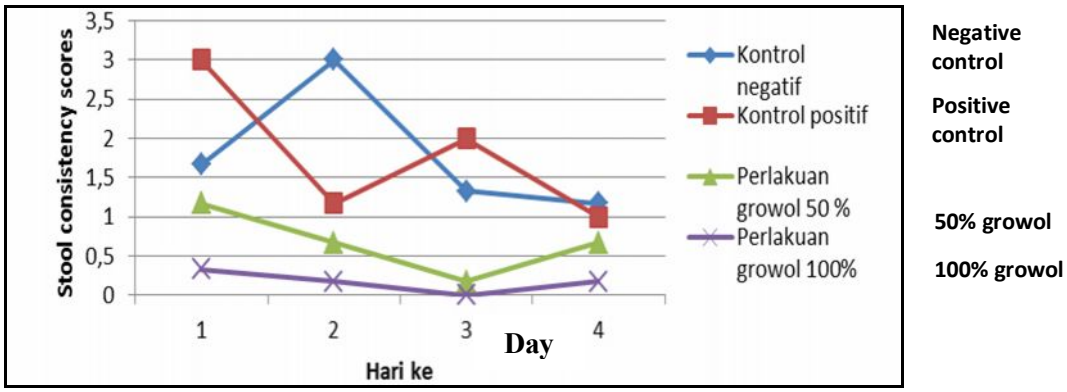

Figure 1. Average of stool concistency scores everyday for 4 days start at day 2 post infection

Figure 2. shows the dynamics of stool consistency during four days of observation. It appears that on first day observations (48 hours after infection of EPEC), the positive control group showed the highest score (3.00) and then declined rapidly until the 4th day of observation. The negative control group experienced a drastic increase on the second day and decreased on day 3 and 4, but it still on the highest scores among other research groups. Growol treatment groups either 50% or 100% seem able to prevent diarrhea which represented by the declining of stool consistency score since day 1 until day 3 but it increased again on day 4, but it still has the lowest score compared to other groups.

Normality test show that the consistency scores were normally distributed (p> 0.05) and homogeneity test (Levene test) indicates that the data are not homogeneous (p> 0.05) to qualify with Two way ANOVA test. Two way ANOVA test results showed significantly different in the average of stool consistency scores between the animal model groups (p = 0.017). It followed by Tukey HSD test and the results are presented in Table 2.

Table 2. Post hoc Tukey HSD of stool consistency scores among animal model groups

Group	P-value	Significancy
NegControl-PosControl	0.989	Not significant
NegControl-TreatmentG 100%	0.028	significant
NegControl-TreatmentG 50%	0.136	Not significant
PosControl-TreatmentG 100%	0.048	significant
PosControl-TreatmentG 50%	0.219	Not significant

In terms of the stool consistency, antibiotics were have been proved not affected to stool consistency until the 4th day of observation (Table 2), although there is a tendency to decrease the stool consistency score close to zero (solid feces) (Figure 2). The treatment of 100% growol has been proved effectively to prevent diarrhea in terms of the consistency of stool. It has been shown from the low consistency scores of the stool from day one to day four observations and statistically proven ~~to~~ have significantly different scores with the negative and positive control groups (Table 2). Consistency scores of 50% growol treatment group seem lower than negative and positive control groups, but the differences were not statistically significant ($p > 0.05$) (Table 2).

Body Weight. Body weight data were observed to obtain additional information on the effect of growol to EPEC infected rat in this study. Weight measurements performed every 2 days during the study (13 days). The average of body weights during study were presented in Figure 3. It appears that administration of 50% growol can increase the body weight of rats during the study and still did not decrease after EPEC infection on day 8 until day 13. Body weight was increase from 212.5 SD ± 15.8 grams in day 1 to 232.5 ± SD 18.00 in the last day of study. Another group is the negative control group, positive control group and 100% growol shows a decreasing of body weight after EPEC infection, even it seem to lose weight before the EPEC infection in 100% of growol treatment compared to rats at other groups (175.3 ± SD 17.2 grams) (Figure 3).

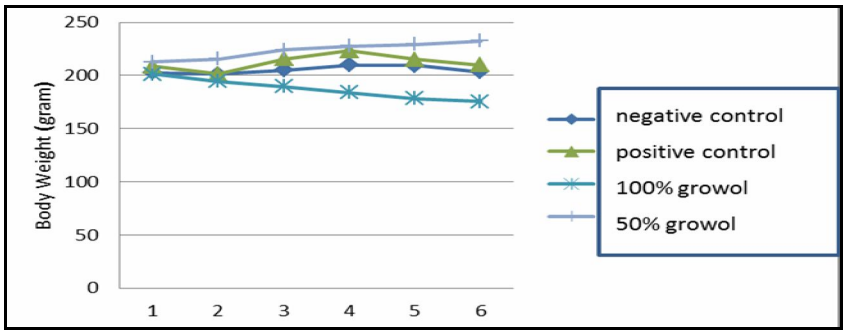

Figure 3. Average of body weight during the study (post infection)

Test for normality and homogeneity (Levene test) of the average weight data indicates that the data were normally distributed and homogeneous ($p > 0.05$) so that it can be done using Two Way ANOVA test. The test results demonstrate the value of $p = 0.000$, which is mean there are significantly differences between the animal model groups. Statistical test is followed by post hoc Tukey HSD test with the results presented in Table 3.

Table 3. Tukey HSD analysis of body weight in average of research groups

Group	P-value	Significancy
NegControl-PosControl	0.383	Not significant
NegControl-TreatmentG 100%	0.002	Significant
NegControl-TreatmentG 50%	0.002	Significant
PosControl-TreatmentG 100%	0.000	Significant
PosControl-TreatmentG 50%	0.077	Not significant

Two way ANOVA test showed that the body weight of rats during the study were significantly different between each groups. Figure 3. and Table 3. show that the body weight of the positive control and negative control group have a similarity which decreased after infected and have no differences statistically ($p = 0.383$). In the treatment group (growol) show a very different pattern. Figure 3. shows that the administration of 100% growol cause body weight of rats decreased during the study and a significantly declined compared to all animal model groups (Table 3). Treatment of 50% growol appears to increase the body weight of rats during the study and did not decrease after EPEC infection. It is statistically significant weight gain compared to the negative control group and the treatment group 100% growol but it is not significantly different compared to the positive control group (Table 3).

Discussion

This study shows that there is a difference between the frequency of defecation from negative control group with all animal model groups ($p < 0.05$). The positive control group was significantly different only with 100% growol treatment group and not significantly different with 50% growol treatment. The same thing happened on stool consistency. Giving 100% of growol was significantly different with the control group ($p < 0.05$), whereas 50% growol was not significantly different with the control group. It means that the administration of 100% growol can prevent diarrhea in rats infected by EPEC. *Lactobacillus* can shorten the duration of diarrhea and shown the positive results for treatment of acute gastroenteritis[9].

Fermented foods were potential containing probiotics, the beneficial bacteria of humans. Probiotics work by inhibiting the growth of pathogenic bacteria in the intestinal mucosa, possibly by competition to hold the attachment of the intestinal epithelium, so bacterial colonization of enterocytes cannot occur[10]. Probiotics can also act as the immune modulator by increasing the number of lymphocytes in spleen[11].

Types of probiotics of *Lactobacillus casei* subsp. rhamnosus has an antimicrobial activity that is able to inhibits the growth of *Staphyloccoccus aureus*, *Escherichia coli, Morganella morganii, Salmonella typhimurium* and *Bacillus cereus*[5]. Process of fermentation in the making of growol for 1-5 days involving lactic acid bacteria dominated by *L. plantarum* and *L. rhamnosus*[6]. Furthermore *L. plantarum* and *L. fermentum* 2B4 2C12 effectively prevent diarrhea caused by EPEC which demonstrated with the increasing of total lactic acid bacteria and the reducing of total *E. coli* in mucosa of the coecum[11]. In vitro study of *L. plantarum* was also shown it could inhibit the growth of *E. coli*[12, 13] and *Staphylococcus aureus* strongly and are against Salmonella typhi[13]. It is necessary further to prove whether the prevention through a mechanism of attachment competition on intestinal epithelial cells by the increasing of total lactic acid bacteria and the decreasing of total *E. coli* in the intestinal mucosa.

In this study, the administration of 100% growol has been proved in reducing body weight, meanwhile giving of 50% growol can significantly increase body weight than the control group ($p < 0.05$). Weight loss in administration of 100% growol indicates that the possibility of a low calorie content in growol. It is necessary to study about the nutrient content in growol. Weight gain in giving of growol 50% will strengthen the research results that the probiotic *L. plantarum* and *L. fermentum* 2B4 2C12 able to increase food intake in rats which exposed to EPEC[11].

Conclusions

The results showed that administration of growol 100% and 50% are effective in preventing diarrhea evidenced by the significant differences in frequency of stool consistency compared to negative and positive control ($P<0.05$). Giving growol 100% seems more potent than 50% in preventing diarrhea but caused weight loss, meanwhile growol 50% treatment will increase body weight even though after rats were infected by EPEC.

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