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STUDY OF VITAMIN D3-FORTIFIED GOAT KEFIR ON PLASMA FIBRINOGEN LEVELS OF DIABETIC RATTUS NORVEGICUS RATS

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ABSTRACT

Background: Diabetes mellitus is often associated with the occurrence of complications. Haemostatic factors, especially hyperfibrinogenaemia, is a common cause of the complication. Goat kefir and vitamin D3 may act as an antioxidant and anti-inflammation agent which can repair pancreatic beta cells.

Objectives: This study aimed to analyse the effect of vitamin D3-fortified goat milk and plasma fibrinogen levels in diabetic rats.

Materials and Methods: This study was an experimental study with pre-post only group design. The samples were 21 male rats divided into four groups; negative control (K-), positive control (K +), treated with unfortified goat kefir (P1), and treated with vitamin D3-fortified goat kefir (P2). The 35-day intervention was conducted, the goat kefir dose was 2 ml/200 g BW/day and the vitamin D dose 600 IU. Fasting blood glucose and plasma fibrinogen were assessed pre- and post-intervention. Blood glucose level was evaluated by GOD-PAP method, while plasma fibrinogen was assessed by Enzyme-Linked Immunosorbent Assay (ELISA) method. The data were analysed with paired t-test and One-Way ANOVA.

Results: There were not significant difference levels of fibrinogen between groups. The intervention groups both showed an insignificant decrease of plasma fibrinogen. The plasma fibrinogen of group treated with vitamin D3-fortified goat kefir went down to 13.47 mg/dl from 16.49 mg/dl (p = 0.49). Meanwhile, the group treated with unfortified goat kefir showed a decrease from 26.81 mg/dl to 24.94 mg/dl (p=0.83). On the other hand, there was a significant decrease in fasting blood glucose in the group treated with vitamin D3-fortified goat kefir from 181.75 mg/dl to 116.25 mg/dl (p=0.03).

Conclusion Our results demonstrate that administration of vitamin D3-fortified goat kefir can decrease fasting blood glucose but not in plasma fibrinogen.

Keywords : Diabetes Mellitus; Fastin blood glucose; Fibrinogen; Goat kefir; Vitamin D3 Fortification

BACKGROUND

Today, diabetes mellitus (DM) has become a global health issue. Type-2 diabetes reported higher prevalence compared to type-1 diabetes and other types of diabetes. According to the International Diabetes Federation (IDF) quoted by Basic Health Survey, the prevalence of diabetes in Indonesia will increase to 6.3% in 2030 from 5.1% in 2000 [1]. World Health Organization (WHO) reported that in 2000 there were 171 million diabetic people, and this will increase to 366 million in 2030 [2].

Diabetes mellitus (DM) is frequently undetected and only come discovered after complications occur [1]. The most common complications are cardiovascular complications. Cardiovascular complications contributed to 50% of death among people with type-2 diabetes [2]. Several studies have shown that haemostatic factors, especially hyperfibrinogenaemia, is associated with atherosclerosis and its complications [3]. Type-2 diabetes associated with peripheral arterial disease affects and increases the concentrations of fibrinogen. [4]

Fibrinogen act as an inflammatory marker that can develop due to the linkage between insulin resistance and vitamin D deficiency [5,6]. Vitamin D deficiency is correlated with metabolic syndrome, cardiovascular disorders, and type-2 diabetes mellitus. Vitamin D stimulates the expression of insulin receptors in peripheral tissues to increase glucose transport [8]. Also, increased insulin sensitivity in response to improved vitamin D status may be due to the suppression of chronic inflammation [9].

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Vitamin D3-fortification in food is needed to increase vitamin D intake among diabetic. Food fortification is an effort to add one or more nutrients that aims to prevent or correct nutritional deficiencies in the population [10]. Goat milk contains more protein, vitamin A, thiamine, riboflavin, niacin, pantothenate, calcium, phosphorus compared to cow's milk [7]. However, some vitamins in goat milk, such as vitamin B6, vitamin B12, vitamin C, vitamin E and folic acid is relatively low, but fermented goat milk had a value of vitamin D3 [7]. A study showed that the consumption of vitamin D3-fortified milk improves levels of 25(OH)D as an improvement in inflammation [11]. At the molecular level, vitamin D is needed to reduce oxidative stress and reduce insulin resistance [12]. Therefore, to increase the nutritional content of goat milk to provide health benefits and reduce inflammation, it must be fortified with vitamin D [7]. Vitamin D3 fortification in goat kefir is necessary due to its low content of vitamin D only at 0.08/100 g [13].

According to Indonesian Dietary Recommendation (AKG) 2019, the recommended dose of vitamin D for 1-64-year-old is 15 µg/day or 600 IU/day.15 Goat milk is an alternative choice for consumers who are allergic to cow's milk. However, the strong aroma and flavour of goat milk caused dome people to dislike it [15]. Besides, milk is easily damaged by microorganisms as it is an excellent growth medium for bacteria and potentially become a medium of spreading pathogenic bacteria. Therefore, to improve the quality and minimize that risk, it is necessary to carry out further processing by fermenting milk into kefir with the help of microbes [16].

Kefir is a product of fermented milk made by inoculating kefir seeds, which consist of bacteria and yeast. Kefir seeds contain natural probiotics, especially *Lactobacillus acidophilus, Bifidobacterium bifidum*, lactic acid bacteria, and yeast, which is an antibacterial, anti-inflammatory, antioxidant, antitumoral, antioxidant and probiotic [17]. In diabetic study showed goat kefir could improves blood glucose and some inflammatory biomarkers [18]. As a probiotic, kefir may weaken the acute phase inflammation characterised by fibrinogen level, which can improve risk factors of chronic diseases such as type-2 diabetes and coronary heart disease [19]. Also, kefir contains vitamin B, vitamin K, folic acid, minerals, amino acids, proteins that are easily absorbed [20.21]. Kefir, as a probiotic drink, may reduce fasting blood glucose and HbA1C in patients with type-2 diabetes mellitus [22]. The administration of 2 ml/day of kefir shows an increase in inflammatory mediators in diabetic rats [23].

Diabetes in rats was determined by measuring fasting blood glucose levels. Rats were declared diabetic if their glucose level exceeded 140 mg/dl [24]. Based on previous studies, daily administration of up to 2ml/200 g body weight of kefir significantly lowered plasma glucose and repair pancreatic β-cells among diabetic rat in the 35-day intervention [24]. As diabetes known as the risk factor for cardiovascular diseases characterised by an increase in plasma fibrinogen, people need to be cautious with this disease. The background of this study was the correlation between diabetes and the likeliness of vitamin D-fortified goat kefir to reduce inflammation. This study aims to determine the effect of administration kefir fortified goat milk with vitamin D3 on fibrinogen levels in diabetic *Rattus Norvegicus* rats.

MATERIALS AND METHODS

This study is an in-*vivo* experiment within clinical nutrition study with a true-experimental method. Pre-post only group design was used with Wistar rats as the sample and data were collected from December 2019 to January 2020. Vitamin D3-fortified goat kefir was made at Integrated Laboratory of Diponegoro University. The experiment was conducted at Animal Trial Laboratory, Faculty of Medicine, Diponegoro University. Fasting blood glucose was assessed at Regional Health Laboratory Hall, Semarang, while plasma fibrinogen was assessed at GAKI Laboratory, Diponegoro National Hospital. Male *Rattus norvegicus* (Wistar rats) were obtained from Farhan Mouse Farm, Semarang.

The sample size was determined using Federer formula, which resulted in seven rats in every group, a total sample of 28 rats. Wistar rats were chosen due to its physiological similarity to human, rapid metabolic rate, are not affected by oestrogen, high adaptability, can be easily cared for, tame, omnivorous, resistant to intervention and unable to vomit thus are easily controlled during the experiment. The dependent variable in this experiment is plasma fibrinogen, while the independent variable is the administration of vitamin D3-fortified goat kefir. The control variable is strain, age, body weight, diet, handling of rats, the environment in which the rats were placed, and also sanitation.

The experiment was started by preparing the rats diet, which was standard rats feed BR-2. The rats were *ad-lib* fed with the following calculation: 6% x body weight (g) = amount of feed (g)/day. They were also *ad-lib* watered. The rats were given seven days to adapt to their cage where temperature and humidity were kept around 25-27°C and 40-70% respectively. During this period, the 12:12 LD cycle was also kept to

help their adaptation process. The cages were cleaned, and the rats body weight was measured daily. After the adaptation period, rats were randomly grouped into four groups: negative control (K-), positive control (K+), given unfortified goat kefir (P1), and given with vitamin D3-fortified goat kefir (P2). The K+, P1 and P2 groups were injected with 230 mg/kg body weight of nicotinamide (NA), diluted in 0.9% NaCl via intraperitoneal. After 15 minutes, 65 mg/kg body weight streptozotocin (STZ) in citrate buffer (pH 4.5) was also injected. These doses are the maximum stable dose of diabetes to be observed in rats [24]. After 15 days of diabetes induction, sample rats were put in fasting for 12 hours. Blood was drawn from plexus retro orbitalis using haematocrit tube. Before and after 35 days of intervention, fasting blood glucose and plasma fibrinogen were assessed. Rats were declared diabetic if the blood glucose >140 mg/dl [24].

Few days after the intervention, a rat from the negative control group (K-) and three Wistar rats from intervention groups (P1 and P2) were found dead. Therefore, at the end of the experiment, there were six rats in the negative control group, seven rats in the positive control group, and four rats each in P1 and P2 groups.

Administration of goat kefir to rats was done based on the previous study that showed that administration of 2 mL/200 g body weight of goat kefir lowered plasma glucose and improved pancreatic β-cells in rats after 35 days. Intervention in this study was carried out for 35 days. The fortified and unfortified goat kefir was made every three days following instruction from the previous study [11].

The collected data contained rats body weight measured every week using a digital animal scale, blood glucose and plasma fibrinogen before intervention as pre-test data, and after intervention as post-test data. Fasting blood glucose was assessed using Glucose Oxidase-Peroxidase Aminoantipyrine (GOD-PAP). In contrast, plasma fibrinogen was determined using Rat Fibrinogen ELISA (Enzyme-Linked Immunosorbent Assay) kit read at 450 nm wavelength by Universal Microplate Reader ELX800.

The collected data was then tested for normality by the Shapiro-Wilk test. Differences on pre- and post- blood glucose and plasma fibrinogen were tested using paired t-test. The difference in all groups was tested by One-Way ANOVA or Kruskal-Wallis test depending on the normality of the distribution (One-Way ANOVA test normally distributed data). The confidence level of the analysis was 95%, or the significance level was 0.05 (p-value). This study has earned an ethical clearance (No. 04/EC/H/FK-UNDIP/I/2020) from the Health Research Ethics Commission (KEPK) Faculty of Medicine Diponegoro University/dr. Kariadi Regional Hospital, Semarang.

RESULTS

Table 1. Baseline Characteristics

Indicators	Groups				
	K- (n = 6)	K+ (n = 7)	P1 (n = 4)	P2 (n = 4)	*p*
Body weight (g)	276.8 ± 23.20	266.1 ± 29.00	255.8 ± 19.29	291.8 ± 18.84	0.202[*]
Fasting blood glucose (mg/dl)	96.98 ± 19.27[a]	155.18±16.33[b]	196.00±46.62[b]	181.75±40.31[b]	<0.001
Plasma fibrinogen (mg/dl)	17.58 ± 4.23	51.57 ± 57.62	26.81 ± 12.99	16.49 ± 6.29	0.089[**]

Value is shown as Mean ± SD
*One-Way ANOVA test **Kruskal-Wallis test
a, b) different notations in the same line showed significant differences in the post-hoc test

Body Weight Characteristics

The changes in body weight during a 35-day intervention is shown in Table 2. All groups showed comparable increase (p = 0.202). K- group showed an increase of 17.83 ± 26.32 g after the intervention. The increase in the control group indicates that glucose metabolism among that group was kept and the rats were healthy. The increase in other groups (K+, P1 and P2) showed diabetes with a sign of polyphagia, occurring because of the lack of glucose intake in cells stimulates the hypothalamus to increase appetite. The difference in body weight between groups after the intervention was significant, with a p-value of 0.034.

Characteristics of Fasting Blood Glucose

Table 2 shows the characteristics of fasting blood glucose levels before and after an intervention. The fasting blood glucose of control group K- and K+ before intervention (96.98 ± 19.27 mg/dl and 155.18 ± 16.33 mg/dl respectively) was increased after 35-day of intervention and there was no significant difference compared to blood glucose level after intervention (113.18 ± 8.59 mg/dl and 208.12 ± 103.24 mg/dl; p>0.05). Although the blood glucose level of the control group increased, the post-intervention mean value of the K-

group was not classified as diabetes mellitus. Meanwhile, P1 and P2 groups shows a significant decrease in blood glucose level pre- (196.00 ± 46.62 mg/dl and 181.75 ± 40.31 mg/dl) and post-intervention (114.35 ± 6.18 mg/dl and 116.25 ± 5.12 mg/dl; p<0.05). If we observed the four treatment groups, the highest difference in blood glucose level reduction (ΔGDP) was shown in the P2 group (p=0.03). This result indicates that the diabetic group treated with fortified goat kefir (P2) was able to reduce blood glucose level significantly compared to the diabetic group treated with unfortified goat kefir (P1).

Table 2. Characteristics of Body Weight, Blood Glucose, and Plasma Fibrinogen Pre- and Post-Intervention

Indicators	Groups				*p*
	K- (n = 6)	K+ (n = 7)	P1 (n = 4)	P2 (n = 4)	
Body Weight (g)					
Pre-	276.8 ± 23.20	266.1 ± 29.00	255.8 ± 19.29	291.8 ± 18.84	0.202*
Post-	294.7 ± 13.26[a]	280.1 ± 16.39[a]	281.5 ± 14.39[a]	307.3 ± 12.53[b]	0.034*
Δ	17.83 ± 26.32	14.00 ± 23.80	25.75 ± 22.57	15.50 ± 28.12	0.896*
p	0.157***	0.170***	0.106***	0.350***	
Fasting Blood Glucose (mg/dl)					
Pre-	96.98 ± 19.27[a]	155.18 ± 16.33[b]	196.00 ± 46.62[b]	181.75 ± 40.31[b]	0.000*
Post-	113.18 ± 8.59[a]	208.12 ± 103.24[b]	114.35 ± 6.18[a]	116.25 ± 5.12[a]	0.004**
Δ	16.20 ± 17.57[a]	52.94 ± 95.22[b]	-81.65 ± 50.04[c]	-65.50 ± 35.44[c]	0.003**
p	0.074***	0.398****	0.047***	0.034***	
Plasma Fibrinogen (mg/dl)					
Pre-	17.58 ± 4.23	51.57 ± 57.62	26.81 ± 12.99	16.49 ± 6.29	0.089**
Post-	20.4 ± 8.32	89.36 ± 202.04	24.94 ± 16.72	13.47 ± 5.29	0.559**
Δ	2.91 ± 8.44	37.79 ± 146.24	-1.86 ± 16.53	-3.02 ± 7.88	0.270**
p	0.437***	0.237****	0.836***	0.499***	

The value was expressed in Mean ± SD
*One-Way ANOVA Test, **Kruskal Wallis Test, ***Paired T-Test, ****Wilcoxon Test
a,b,c,) Different notations on the same line indicate significant differences

Characteristic of Plasma Fibrinogen

Table 2 indicates that there was an increase of plasma fibrinogen in control groups K- and K+ (Δ = 2.91 ± 8.44 mg/dl and 37.79 ± 146.24 mg/dl respectively). On the contrary, the plasma fibrinogen level in treatment groups P1 and P2 declined after intervention. The difference observed in the treatment group was -1.86 ± 16.53 mg/dl and -3.02 ± 7.88 mg/dl respectively. The highest decline was shown in delta group P2 (Δ = -3.02 ± 7.88 mg/dl), and there was no significant reduction of plasma fibrinogen level pre- and post-intervention (p = 0.49). The observed result indicates that the diabetic rats given vitamin D3-fortified kefir has stronger anticoagulant activity compared to unfortified kefir, which was shown in the decreased level of plasma fibrinogen after 35day intervention. Based on *One-Way ANOVA* test, there was no significant difference in plasma fibrinogen levels in all groups before and after an intervention (p = 0.27).

DISCUSSION

This study aims to determine the association between vitamin D3-fortified goat kefir on plasma fibrinogen level in diabetic rats. The results showed that vitamin D3-fortified goat kefir was statistically able to reduce plasma fibrinogen and blood glucose levels in diabetic rats. Diabetes was administered to the rats through the injection of STZ-NA. The control and treatment groups of diabetic rats developed hyperglycemia due to autoimmune mechanisms. This autoimmune mechanisms generated reactive oxygen species (ROS). ROS exceeding antioxidant levels induce oxidative stress. Oxidative stress reduces the immunes responses through activation of nuclear factor kappa beta (NfKB) and activating protein 1 (AP-1), lipid peroxidation, increased production of proinflammatory cytokine, and pancreatic B-cell damage. Imbalance between free radical production and antioxidant production leads to necrosis of pancreatic B-cells [28]. Furthermore, this condition will increase blood glucose level due to hyperglycaemia and affecting the inflammation status of the study sample. The inflammatory reaction that occurs in the early phase starts from neutrophils then macrophages enter the injured tissue. These cells will produce ROS, which has detrimental effects on surrounding tissues. Excessive production of ROS can cause tissue damage, haemostasis and interfere with the coagulation process. This condition makes the formation of plasma fibrin during the coagulation process takes a longer time to be dissolved and degraded. Increases in plasma fibrinogen, factor VII activity, and

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plasminogen activator inhibitor (PAI) cause increased platelet aggregation and enhanced activation of plasma fibringen [29, 30].

In this study, K+, P1, and P2 group diabetics have plasma fibrinogen levels that are generally elevated in acute infections, are directly proportional to the hyperglycemic state in patients with diabetes mellitus, and are closely associated with the development of thrombosis [31]. Moreover, the results of the study using STZ-induced rats found that the longer duration of acclimatisation and the age of adult rats affecting fasting blood glucose and plasma fibrinogen levels [32]. The diabetic condition causes an increase in platelets due to thrombotic hyperactivity. Consequently, the activation of the prothrombotic coagulation factor increased, whereas fibrinolysis decreased. This circumstance explained the decrease in fasting blood glucose level in the current study sample, but the fibrinogen levels depletion was not significant. Another study found that the anti-diabetic effect of bacteria contained in kefir was able to significantly reduced blood glucose, HbA1c, and phosphate levels in patients with type-2 diabetes during the 10-week intervention. Similar studies found that administration of 2 ml/day of kefir causes an increase in inflammatory mediators on diabetic rats [25, 33]. Also, it has been reported that kefir may reduce glycemia and improves the balance of pro-inflammatory and anti-inflammatory cytokines [22, 34]. These findings were similar to the results of the current study, where the fasting blood glucose level of the intervention group decreased. In contrast, the plasma fibrinogen levels of the treatment groups indicate no significant differences before and after treatment. The occurrence of hyperglycaemia found in rats was affecting the haemostatic state and coagulation; thus, it stimulates excessive fibrinogen production [31]. The role of kefir as a probiotic and antioxidant administered in a diabetic situation directly functions as a fibrinogen polymerisation protector, and thereby it would accelerate the wound healing by preventing oxidative stress which can inhibit coagulation [21, 33, 35]. Several studies found the link of a high level of antioxidant activity in kefir to malondialdehyde (MDA) levels. MDA levels are used in measuring free radical activity. Kefir was reported to be able to reduce the MDA levels in diabetic rats [21].

Improvement in blood glucose and plasma fibrinogen levels in each treatment groups that received kefir showed that the probiotics contained in goat kefir have beneficial effects on type-2 diabetes treatment. The anti-diabetic effect from Lactobacillus and Bifidobacterial activity in kefir can reduce blood glucose levels in diabetic rats by stimulating glycogen formation in the liver from blood glucose and antioxidant status [36, 37]. The antioxidant status is directly influenced by oxidative stress, which occurs early in the development of diabetes. Diabetes condition causes insulin failure to stimulate glucose uptake by fat and muscle tissue, resulting in a high concentration of glucose in the blood. This condition results in oxidant products increment and causing damage to the defence triggered by antioxidant [37]. In the current study, the antioxidant status was not measured, but the decrease in fasting blood glucose was related to the antioxidant activity contained in kefir. The decline in blood glucose is also triggered by gut microbiota that produced insulinotropic polypeptides and glucagon, stimulating glucose uptake into muscles [22].

Meanwhile, the administration of vitamin D increases the sensitivity of insulin secretion when blood glucose levels are elevated. It modulates Peroxisome Proliferator-Activated - γ (PPAR-γ) signalling in glucose metabolism and inflammation process, also increase PPAR-γ expression during adipogenesis [33]. Vitamin D affects the ß-cells function and mass by increasing the proliferation of pancreatic ß-cells so that the ß-cells mass increases. The dose given to the P2 group was 600 IU (15 µg) of vitamin D3 based on the Recommended Dietary Allowances (RDAs) and The Indonesian Dietary Recommendation (AKG) 2019. A study found that the administration of high doses of vitamin D3 per-oral was able to delay the development of disturbances in high fasting blood glucose values in diabetic subjects, with the same dosage and concentration being carried out in experimental rats [38, 39]. The result of the study illustrated that vitamin D3 could increase calcification in blood vessels and stimulate proliferation of smooth-muscle cells in blood vessels. One factor that might distinguish the control group from the treatment group is the differences in the immune response of the rats. The rats that have low immunity have a higher chance of getting an infection, so they are susceptible to inflammation due to elevated fasting blood glucose even in condition without treatment [39]. This finding is in line with the development of diabetes mellitus in humans so that the dose of vitamin D3 is safe for use in experimental animals [38]. Based on the results of the study, vitamin D3-fortified kefir had a more significant effect on lowering fasting blood glucose levels in diabetic rats.

Moreover, there was a decrease in fibrinogen levels, accompanied by a decline in blood glucose levels in P1 and P2 groups. The low level of plasma fibrinogen in P1 and P2 groups indicated a reduction in inflammation and a decrease of disease severity in people with diabetes mellitus. Lactobacillus found in kefir activates innate immune receptors, named as toll-like receptors (TLRs) that are involved in activating pro-

inflammatory cytokines (TNF-a, IL-6). The IL-6 has a role in stimulating fibrinogen synthesis that occurs in the liver. The role of fibrinogen as a biomarker of inflammation is to carry out the coagulation process to maintain the haemostatic system [24, 40].

Fibrinogen levels was associated with glucose metabolism including fasting blood glucose and markers of diabetes mellitus type 2 [25]. A study found that a group of diabetic rats experienced a significant increase in fibrinogen levels due to streptozotocin injection [26]. The results of the fibrinogen measurement in the experimental rats recorded 0.93±0.46 mg/ml of fibrinogen. This increase lasted until the 96th hour, with fibrinogen level in 1.80±0.1 mg/ml. The study found that fibrinogen level increased as diabetes progressed [26]. The increase in fibrinogen level to the inflammation stage in diabetic rats ranged from 93 to 180 mg/dl. Based on these findings, all groups of diabetic rats had not yet reached the inflammatory stage. Based on a study observing the old category of type-2 diabetes towards fibrinogen, the highest duration of Type-2 Diabetes in patients was in the more than ten years category with a mean value of 690.9 mg/dl [41]. Also, in the results, the number of research samples that differed between the group was prone to bias. This is due to several samples of rats that died during the study.

The vitamin D3-fortified goat kefir affects the haemostatic state of diabetic rats, showing a lower fibrinogen result in P2 group. The intake of vitamin D-fortified goat kefir triggers high antioxidant activity in cell protection so that the inflammation does not occur [42]. The addition of vitamin D3 in goat kefir triggers the production of cytokines (IL-10.TNF-α, dan IL-6) and activates the nitric oxide (NO) release from the blood cells. The release of NO can be beneficial in inhibiting the atherogenic monocytes and LDL infiltration to the arterial walls. Also, the release of NO acts in inhibiting platelet aggregation and genes expression involved in atherogenesis [43]. To summarise, the role of goat kefir in this study is in line with the existing evidence from previous studies, which reported that goat kefir could prevent tissue damage in diabetic rats even though the results are not statistically significant.

CONCLUSIONS

Administration of 2 mL/200 g body weight/day of vitamin D3-fortified goat kefir (vitamin D3 fortification dose of 600 IU/day) lowers plasma fibrinogen among diabetic mice insignificantly.

ACKNOWLEDGMENT

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