EFFECT OF HIGH SALT (SODIUM CHLORIDE) INTAKE ON FRACTURE RISK IN MALE WHITE RATS (*Rattus norvegicus*)
An Experimental Laboratory Study

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ABSTRACT

The past epidemiologic studies did not have definite explanation about the effect of consuming high salt (sodium chloride) on fracture risk and they just showed that high salt (sodium chloride) diet influenced bone density. The objective of this study was to explain the effect of high salt (sodium chloride) intake on fracture risk. Experimental animals used in this study were 40 three-month old male Wistar strain white rats. Those rats were randomly divided into four groups, which consisted of one control group and three treatment groups. The control group were given orally 2 ml of distilled water/200 g BW/day, while the treatment groups were subjected to three salt (sodium chloride) suspension orally of 72 mg/200 g BW/day, 144 mg/200 g BW/day and 216 mg/200 g BW/day, respectively, all of which were dissolved in 2 ml of distilled water. After 8 weeks, the bone density was measured at the metaphysial part of femoral bone of all experimental animals using ultrasound densitometry DBM Sonic 1200, the cortical width with radiograph, and the bone strength with Unconfined Compressive Strength Machine Modified. The result was subsequently presented descriptively and analyzed using multivariate analysis and discriminants analysis. It showed that all of the experimental animals with high salt (sodium chloride) intake had a significant difference from control ($p < 0.05$). Pairwise comparison showed that significant difference ($p < 0.05$) occurred between the treatment groups of 72 mg and those of 144 mg and 216 mg. The significant difference was also found between the group of 144 mg and that of 216 mg. The tests of Equality of Group Means showed that the significant difference occurred in the response of bone density, bone width and bone strength of all groups ($p < 0.05$). The stepwise statistics revealed that the response of bone density will appear firstly followed by the response of bone width in associated with high salt (sodium chloride) intake ($p < 0.05$). Linear regression analysis resulted in linear equation, showing that with increment of every 1 mg of salt (sodium chloride) administration, there would be a decrease in the sound wave velocity through the bone of about 0.478 m/second, a decrease in cortical width of about 0.001 millimeter and decrease in bone strength of about 0.416 Newton. In conclusion, high salt (sodium chloride) intake would cause increase in fracture risk and the increase of its doses administration would be followed by further increase in fracture risk. However, further studies with different methods and measurement devices are needed so as to identify the doses of salt (sodium chloride) intake that can increase the fracture risk.

Keywords: *salt (sodium chloride) intake, bone strength, bone density, fracture risk.*

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INTRODUCTION

Osteoporosis is the most common bone abnormalities both in western countries and the United States (Maddison 1998). In the US, in 1999 there were approximately 1.3 billion bone fracture cases that related with osteoporosis, requiring a cost of more than US $ 10 billion a year (Tjokroprawiro 2003). In Indonesia, however, there has been no comprehensive and in depth studies on osteoporosis, either on its incidence or effects (Roeshadi 1997; Tjokroprawiro 2003). Nevertheless, Roeshadi (2002) found that the incidence of osteoporosis in Surabaya was 26% among post-menopausal women. Osteoporosis, according to Consensus Development Conference 1991, is defined as a disease characterized by low bone mass, microarchitectural deterioration of bone tissue leading to enhanced bone fragility, and a consequent increase in fracture risk (Maddisson 1998). This definition has been completed by WHO in 1994. The additional definition is based on bone density, in which bone density of osteoporotic patients is less than 2.5 standard deviation

of bone mass in peak period (Maddison 1998; Roeshadi 2000).

One attempt to prevent early osteoporosis is through diet regulation by controlling natrium intake (Tjokroprawiro, 2003). Matkovic et al (1996) found an increase of urine calcium excretion as much as 50 mg/day occurs in the addition of natrium intake of 500 mg/day. A study by Sellmeyer (2002) revealed increase in calcium excretion and signs of bone resorption increase in women randomly receiving high-natrium diet of 225 mmol/day. Whereas, Evans et al. (1997) found an increase of bone resorption signs resulting from the increase of natrium intake from 300 mmol/day as compared to 50 mmol/day. In his study, Blackwood (2000) revealed that urinary calcium loss due to increasing natrium diet of 100 mmol/day for 10 years will be equal to to 10% of total calcium in the body.

In a study by Melton and Riggs (1993), in the field of biomechanics, the occurrence of bone fracture depends on three factors, 1) bone geometrical structure, 2) the composition of bone-constituting substances, represented by bone mass density, and 3) location and the direction of working force. This confirmed a study by Watts (2003) who found that bone density cannot be applied as a guidance to identify the reduced risk of non-vertebral bone fracture. It should be confirmed with other markers, since the increase of bone mass density can only count 6-12% of the reduced risk of non-vertebral bone fracture. Barzel (1997) and Lin (2001), as cited in the paper of Sacks (2001), proposed that there was difference between control and group that received natrium diet, either in the reduction of bone formation or resorption. However, in general, they found that natrium diet had no significant effect on bone metabolism (Barzel 1997).

Since the effect of high natrium diet on bone density remains controversial, and due to the presence of human confounding factors that were omitted in previous studies, a study employing another method was required to identify the effect of high salt (NaCl) diet on bone density and its correlation with the risk of bone fracture. According to Burhanuddin (2001), the need of salt consumption among Indonesians is increasing annually, along with the increase of population and preserved dried food products that need the use of salt. In 2000, salt consumption among Indonesians was 3 kg/person/year or 8.2 g/person/day. Favus (1993) found that normal daily salt consumption was 10 mmol - 350 mmol/person/day or 230 mg – 8.050 mg/person/day.

The objective of this study was to prove the effect of high salt (NaCl) diet on bone density and cortical bone thickness as well as to define correlation between high salt (NaCl) diet and the increasing risk of bone fracture. This study was particularly aimed to prove that high salt diet causes the reduction of bone density and thickness as well as to find the effect of increased salt diet on the risk of bone fracture. If high salt diet does affect the reduction of bone density and thickness and the risk of bone fracture, limitation of salt consumption for public can be advised to prevent early emergence of osteoporosis.

MATERIALS AND METHODS

This study was a laboratory experimental study using post-test only control group design (Zainuddin 2000). Sample population in this study comprised male white rats taken from Experimental Animal Unit, Gadjah Mada University, Yogyakarta. Rats selected should meet some criteria: Wistar strain *Rattus norvegicus* (white rats), male, about 3-month old, with body weight around 200 grams and healthy, as marked by active movement and bright eyes. Sample comprised 10 rats for each treatment group, so that total sample size was 40 rats. Samples were taken from the population using simple random sampling. All samples were divided randomly into four groups: Group 1 : Control group white rats without certain treatment, only receiving distilled water 2 ml/200 mg BW/day, Group 2, white rats receiving NaCl diet of 72 mg/2 ml distilled water/200 mg body weight/day, Group 3 : white rats receiving NaCl diet of NaCl 144 mg/2 ml distilled water/200 mg body weight/day, and Group 4 : white rats receiving NaCl diet of NaCl of 216 mg/2 ml distilled water/200 mg body weight/day. Treatment was given every morning at the same hour, 7 a.m. Acclimatization of the animals was conducted for seven days in laboratory setting, in which standard feed and drinking water were given ad libitum. The scaling was conducted in the morning before the first treatment using Torbal scale (Torsion Balance).

Bone density was measured by femoral bone metaphysis after the animals were anesthetized. Measurement was undertaken four times using Ultrasound DBM Sonic 1200, and the result was recorded in m/second, and the mean values were taken. Cortical bone thickness was measured as a difference between diaphysical external diameter of femoral cortical bone and internal diameter right on the middle of the diaphysis in millimeter from antero-posterior x-ray in 4 x magnification. The results were recorded and the mean was taken. Bone strength measurement was performed after all muscles and tendons were cleaned from femoral bones. The femoral bones were placed on Unconfined Compressive Strength Machine Modified, and force was applied until the bones were broken, and

the applied force was recorded. The result was expressed in Newton. This study was performed at the Department of Biochemistry, Airlangga University School of Medicine, Radiology Installation, Airlangga University School of Medicine, Dr Soetomo Hospital, and Laboratory of Land Mechanics, Civil Engineering, Sepuluh November Institute of Technology, Surabaya. The study was conducted for five months.

RESULTS

Data of the results were analyzed using descriptive statistics in order to obtain better feature on data distribution and standard deviation of the data. The result of descriptive statistics on bone density, cortical bone thickness and bone strength can be seen in Table 1.

Table 1. Mean and standard deviation of bone density (meter/second), cortical bone thickness (millimeter), and bone strength (Newton) from control and treatment groups

Groups	Variables	Mean	SD
Control	Bone density	1707.000	40.383
	Bone thickness	0.9589	0.0394
	Bone strength	664.111	17.446
NaCl 72	Bone density	1667.000	27.898
	Bone thickness	0.9079	0.0412
	Bone strength	609.714	8.499
NaCl 144	Bone density	1635.500	22.157
	Bone thickness	0.8610	0.0317
	Bone strength	589.900	15.702
NaCl 216	Bone density	1602.500	24.897
	Bone thickness	0.8330	0.0312
	Bone strength	570.500	21.267

The result of descriptive statistics on the response of bone density, cortical bone thickness, and bone strength, is displayed in Table 2.

Table 2. Mean and standard deviation of the response of bone density response (meter/second), cortical bone thickness (millimeter) and bone strength (Newton) of the treatment groups.

Groups	Variables	Mean	SD
NaCl 72	Bone density response	40.000	27.898
	Bone thickness response	0.0510	0.0412
	Bone strength response	54.397	8.499
NaCl 144	Bone density response	71.500	22.157
	Bone thickness response	0.0979	0.0317
	Bone strength response	74.211	15.702
NaCl 216	Bone density response	104.500	24.897
	Bone thickness response	0.1259	0.0312
	Bone strength response	93.611	21.267

Based on bone density, there was a highly significant different mean between control and the whole treatment groups with $p = 0.020$ for 72 mg NaCl, with $p = 0.000$ for each 144 mg and 216 mg NaCl ($p < 0.05$). There was a highly different mean between treatment group with 72 mg NaCl and treatment group with 144 mg NaCl ($p = 0.043$; $p < 0.05$), and there was significant different mean from treatment group with 144 mg NaCl ($p = 0.000$; $p > 0.05$). Between treatment group with 144 mg and that with 216 mg also had significant difference with $p = 0.020$ ($p > 0.05$).

Regarding the thickness of cortical bone, there was a highly significant different mean between control group and the whole treatment groups with $p = 0.009$ for 72 mg NaCl, and $p = 0.000$ for each 144 mg and 216 mg NaCl ($p < 0.05$). There was also highly significant different mean between treatment group with 72 mg NaCl and that with 144 mg NaCl ($p = 0.013$; $p < 0.05$), as well as with treatment group receiving 216 mg NaCl ($p = 0.000$; $p > 0.05$). However, treatment group with 144 mg NaCl and that with 216 mg NaCl showed no significant different mean with $p = 0.089$ ($p > 0.05$).

In regard with bone strength, there was highly significant different mean between control group with all treatment groups with $p = 0.000$ ($p < 0.05$). There was highly significant different in mean between treatment group receiving 72 mg NaCl with that receiving 144 mg NaCl ($p = 0.019$; $p < 0.05$), as well as with the treatment group receiving 216 mg NaCl ($p = 0.000$; $p > 0.05$). Treatment group receiving 144 mg and that receiving 216 mg NaCl showed significant difference with $p = 0.063$ ($p > 0.05$).

The variables of bone density response, cortical bone density response, and bone strength response revealed highly significant difference in mean response in the interaction between dose groups with $p < 0.05$, excluding the response of bone thickness between treatment group receiving 144 mg NaCl and that receiving 216 NaCl, which had no significant difference with $p = 0.079$ ($p > 0.05$). Subsequently, from the whole result of Equality of Group Means test, there was a highly significant difference in response value between NaCl administration and the response of bone density, cortical bone thickness and bone strength with $p = 0.000$ ($p < 0.05$) for bone density and bone strength responses and $p = 0.001$ ($p < 0.05$) for bone thickness response. The use of stepwise statistics revealed that the response of bone density emerged first, followed by the response of cortical bone thickness ($p < 0.05$).

Table 3. Result of discriminant test using Stepwise statistics between the response of bone density, cortical

bone thickness, and bone strength in various NaCl doses (mg).

Variables	Wilks' Lambda	p
Bone density response	0,471	0,000
Bone thickness response	0,253	0,000

The magnitude of change response in bone density (meter/second), cortical bone thickness (millimeter), and bone strength (Newton) resulting from the administration of NaCl diet could be identified from linear regression analysis of each variable in order to predict the effect of NaCl on bone density, cortical bone thickness, and bone strength. The result of the linear regression analysis can be seen in Table 4.

Table 4. Result of linear regression analysis on bone thickness (meter/second), cortical bone thickness (millimeter), and bone strength (Newton).

Variables	Regression constant	Regression coefficient	P
Bone density	1704.870	-0.479	0.000
Bone thickness	0.954	-0.001	0.000
Bone strength	653.791	-0.416	0.000

It can be seen that the p value was 0.000 ($p < 0.05$), indicating that the administered NaCl solution had significant effect on bone density. The estimating equation obtained from the regression analysis was as follows:

Bone density = 1704.870 - (0.479 X NaCl dose)

in which bone density was in m/second and NaCl dose was in mg. The constant of 1704.870 indicates that if there was no treatment of NaCl solution administration (x = 0), the expected result of bone density measurement in experimental animal was 1704.9 m/sec. The regression coefficient of -0.479 indicated that each NaCl solution dose increment of 1 mg would reduce the rate of sound wave in bone of 0.479 m/second.

Cortical bone thickness = 0.954 - (0.001 x NaCl dose)

in which cortical bone thickness was in mm and NaCl dose was in mg. The constant of 0.954 indicated that if there was no treatment of NaCl solution administration (x = 0), the expected result of cortical bone thickness measurement in experimental animals was 0.954. The regression coefficient of -0.001 indicated that each NaCl solution dose increment of 1 mg would reduce cortical bone thickness of -0.001 m/second.

Bone strength = 653,791 - (0.416 X NaCl dose)

Bone strength was stated in Newton and NaCl dose was mg. The constant of 653.791 indicated that if there was no treatment of NaCl solution administration (x = 0), the expected result of bone strength measurement in experimental animals was 653.8 Newton. The regression coefficient of -0.416 indicated that each NaCl solution dose increment of 1 mg would reduce bone strength in resisting force of 0.416 Newton. From those three regression equations, it is apparent that each increment of NaCl solution may result in the reduction of bone density, cortical bone thickness, as well as bone strength in resisting force. The measurement of each variable after various NaCl doses administration can be seen in Figures 1, 2, and 3.

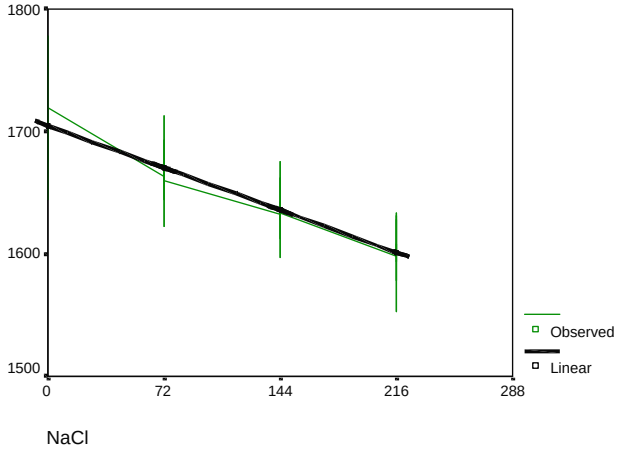

Figure 1. Correlation between NaCl dose and bone density

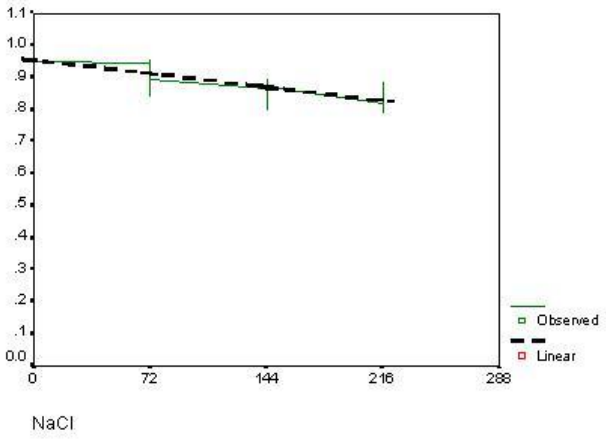

Figure 2. Correlation between NaCl dose and cortical bone thickness

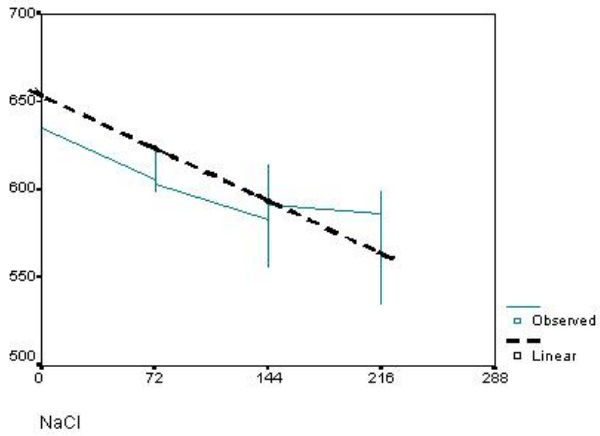

Figure 3. Correlation between NaCl dose and bone strength

DISCUSSION

It has been widely proved that the administration of high salt (NaCl) diet may have effect on bone quality and quantity. Most of the authors stated that the effect of high salt diet administration primarily results in the reduction of bone density without mentioning the magnitude of the reduction. The reduction of bone density leads to decreasing strength of the bone in resisting force that can be accommodated. Therefore, the bone may have a high risk of fracture. According to Melton and Riggs (1993), in addition to bone quality and quantity, other factors that determine bone strength in resisting force to prevent bone fracture are geometric shape and the magnitude of the impacting force. This study was aimed to identify the effect of high salt diet administration on both factors that affect the bone strength, i.e., bone density and geometric shape of the bone, which was represented by cortical bone thickness, as well as to directly identify the bone strength itself.

The effect of high salt (NaCl) diet administration on those three variables was verified with the following tests. Multivariance statistical test was used to find different effect of treatment between groups on bone density, cortical bone thickness, and bone strength. The result of this statistical test revealed that there was significant difference in the mean of the measurement results, either in bone density, cortical bone thickness or bone strength between the groups with $p = 0.000$ ($p < 0.05$) (Pitono 2002).

The result of Manova test supported the findings of previous researchers that high salt diet resulted in the reduction of bone density, as has also been pointed by Sellmeyer (2002) that there was an increase of calcium excretion and increased signs of bone resorption in women randomly given with high natrium diet. Furthermore, Blackwood (2001) suggested that the increase of urine calcium loss due to excessive natrium intake could not be compensated by intestinal calcium absorption and tended to reduce the ionized plasma calcium.

The increase of bone resorption started from the activation of hormonal compensatory mechanism to restore plasma calcium level through the increase of hormone secretion that stimulates bone resorption activation, the PTH (Bushinsky 2001). The increase urine natrium and calcium excretion due to high salt intake commences from the increase of renal filtration rate that may directly cause stimulation to macula densa to activate Renin-Angiotensin-Aldosterone system. The outcome of the system is the increase of natrium excretion to maintain plasma calcium concentration (Guyton 1997).

The increase of urine natrium and calcium excretion occurs due to several mechanisms. One of these mechanisms are inhibited formation of natrium and calcium-loading protein at the luminal side of renal tubular cells, which results in the decrease of permeability to natrium and calcium, the increase of peritubular capillary hydrostatic pressure that causes the decrese of natrium and calcium reabsorption, as well as the reduction of Na-K ATPase pump activity in renal proximal tubule resulting in the reduction of natrium and calcium reabsorption. The final outcome of those three processes is the increasing excretion of both natrium and calcium (Ganong 1999; Ganry 2003). High excretion of urine calcium results in disordered calcium balance in the plasma, which subsequently results in the increase of hormonal regulation by PTH release into the plasma (Blackwood 2001).

Subsequently, PTH may re-regulate plasma calcium balance through several mechanisms. First, the increase of 1.25 dihydroxy calcitriol production, which may enhance calcium absorption within intestinal lumen (Ganong 1999). Second, the inhibition of expression and synthesis of matrix protein in osteoblasts, which results in osteoblast retraction at bone surface through proteolytic modification of osteoblast cytoskeleton (Sasaki 1996). Third, to control osteoclast activity through stromal cells and osteoblasts that control osteoclast differentiation. Such differentiation is regulated by association among those cells through osteoprotegerin (OPG) synthesis and ligand for NF-kappaB activator (RANKL = Receptor Activator of Nf-Kappa Signaling-Ligand/OPGL) (Oshiro 2002; Shiotani 2002). If RANKL is bound to RANK in osteoclast, these cells become activated to differentiate and

proliferate. This process undergoes by the help of M-CSF (macrophage colony stimulating factor) through its activation of pre-osteoclast fusion to become osteoclast and the increasing formation of ATPase proton pump, while osteoprotegerin acts by inhibiting bone resorption process through its inhibition of ruffled border formatin (Mulari 2003). Fourth, the reduction of bicarbonate ion reabsorption, the reduction of Na-K ATPase pumping activity, and the reduction of co-transport protein for natrium and calcium (Razaq 2000). Another mechanism, which results from increased plasma natrium level due to high calcium diet, is elaborated by Arnett (1996), in which the change of resorption activity in osteoclast may occur due to extracellular pH alteration, even though such alteration is small, approaching physiological pH. All of those processes finally lead to the reduction of bone density which results in reduced capability of bone to maintain load.

From regression analysis, it was found that the effect of high salt (NaCl) diet on each dependent variables could be identified from several regression equations, i.e., Bone density = 1704.87 – (0.479 x salt (NaCl) mass), cortical bone thickness = 0.954 – (0.001 x salt (NaCl) mass), and bone strength = 653.791 – (0.416 x salt (NaCl) mass). Each constant indicates the measurement unit that was expected to be obtained when no treatment is given to the experimental animals. In other words, if the experimental animals were not given with salt (NaCl) diet in addition of the normal one, the bone density rate would be 1704.87 m/sec, cortical bone thickness was 0.954 millimeter, and bone strength to resist load until the occurrence of fracture of 653.791 Newton. This supported the results of previous studies, such as that by Matkovic or Massey and others, who showed clearly the increase of urine calcium excretion of 1 mmol in each 100 mmol increment of urine natrium.

From the result of comparative analysis using pairwise comparison test we found that the mean of bone density showed highly significant difference between control group and the whole treatment groups ($p < 0.05$). Similarly, there was also highly significant difference between treatment group receiving 72 mg NaCl and that with 144 mg and 216 mg NaCl ($p = 0.000$; $p < 0.05$). Significant different mean ($p > 0.05$) was also found between treatment group with 144 mg and that with 216 mg. Similarly, comparative test using pairwise comparison test revealed that the mean of the response of bone density, cortical bone thickness, and bone strength were different between control group and whole treatment groups. Different mean was also found between the administration of 72 mg NaCl and 144 mg and 216 mg NaCl, and between 144 mg NaCl and 216 mg NaCl ($p < 0.05$). This indicates that the different effect of NaCl on bone density, cortical bone thickness, and bone strength is also determined by the doses given.

Linear regression analysis revealed reduction either in bone density, bone thickness and bone strength in each increment of salt (NaCl) administration. However, not all reduction result in the difference in the mean of bone density, thickness, and strength. This confirmed the study by Lees (2000) that there is different regulation in the resorption activity between osteoclast with few nucleus and that with multiple nucleus, which results from pH changes around the osteoclast. Krieger (1992) also found similar findings that pH changes around the osteoclast may result in the change of resorption activity of the osteoclast. Based on the studies on high salt (NaCl) diet, most of the experts suggest limiting salt (NaCl) consumption as much as 2 grams daily to prevent negative effects on the bone due to high salt (NaCl) consumption.

CONCLUSION

The administration of high salt (NaCl) diet may reduce bone density and cortical bone thickness. Increased salt (NaCl) diet consumption may increase the risk of bone fracture. Similar study should be performed using another method, i.e., the measurement using histomorphometry and biochemistry as a comparison. Furthermore, a biomolecular advanced studies should also be performed on bone cells, for example, to the cytokines in the bone, by providing the same treatment, so that the effect of salt (NaCl) on bone health can be elaborated further. Further studies are also needed on the effect of salt (NaCl) administration in more various dose increments, so that it can be found in which dose bone density and strength start to decline.

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