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Low Intake of Methionine Increases the Risk Cartilage Damage of the Knee Joint through DNA Methylation of the IL-1β Gene and the Expression of IL-1 β

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ABSTRACT

Low intake of methionine lowers the quality of cartilage and chondrocytes modulate increases the secretion of protease and degeneration of cartilage. Exposure to low intake of methionine has great potential to decrease DNA methylation that can increase gene expression catabolic joint cartilage. The purpose of this study to prove a low intake of methionine (DL-methionine 0.00%) can decrease DNA methylation gene IL-1β and increase the risk of damage to the joint cartilage in rabbits *New Zealand (Oryctologus* cuniculus). Adult female rabbits were divided into 6 groups: three groups (1,2,3) normal rabbit and the next three groups (4.5 and 6) diincisi rabbit ACL. Formula DL-methionine were classified by the addition of DL -metionin per kg formula the addition of DL-methionine 0.25%; DL-methionine 0.15% and 0.00% DL- methionine. Rabbits will undergo acclimatization period of 7 days with standard polar food and randomly followed by administration of the formula DL-methionine for 35 days. Measurement of body weight once a week and every day eating left over measurements to calculate servings eaten per day per rabbit. After 5 weeks, the rabbits were sacrificed blood and tissue taken joint cartilage. Blood samples for examination of DNA methylation gene IL-1β methods and levels of serum IL-1βmethod *ELISA* using a commercial kit *RayBio* IL-1βRabbit. The tissue samples cartilage of the knee joint for examination of the expression of IL-1β immunohistochemical methods with the primary antibody anti-rabbit IL-1β using a commercial kit *Santa Cruz* (Sc7884) and damage to joint cartilage stained with H & E. The results of the study low intake of methionine (0.0% DL-methionine) can decrease DNA methylation gene IL-1β was significantly ($P < 0.05$), increased levels of IL-1 β serum ($p < 0.05$), and increased expression of IL-1 β ($p \leq 0.05$), and severity of knee joint cartilage. In conclusion low methionine intake has the potential of increasing the breakdown of joint cartilage through a reduction in DNA methylation gene IL-1 β and increased expression of IL-1 in the cartilage and the level of damage to the articular cartilage of the knee.

KEYWORDS: Low Methionine, DNA methylation gene IL-1β, destruction, joint cartilage

INTRODUCTION

When it is known that the pathogenesis of osteoarthritis significantly occurred because epigenetic changes, especially DNA methylation [3]. The cartilage of epigenetic changes cause changes in gene expression of cytokines, proteinase, extracellular matrix proteins [4,5,9,18]. Some research indicates there has been a deviation multiple gene expression catabolic such as IL-1β,*matrix metalloproteinases* (MMP), aggrecanase (ADAMTS-4, ADAMTS-5) and a number of other cytokines in cartilage of osteoarthritis [1,2, 19]. In epigenetic mechanisms of dietary factors have a strong and active effect changed the pattern of epigenetic at the end of modifying phenotypes [8]. Methionine is one of essential amino acids in protein synthesis is key, growth , normal development and a major source of methyl groups (CH3) and sulfur [24]. In the body methionine is converted to S-adenosy methionine (SAM) as the main donor for methyl endogenous DNA methylation. Methionine deficiency inhibit the formation of SAM causing the modification of DNA methylation and gene expression change.

In patients with osteoarthritis are known to have DNA catabolic genes such as IL-1β and gene expression of IL-1β gene in cartilage tissue increased [17, 11]. Interleukin -1β as major inflammatory mediators that can destroy joint cartilage, interleukin-1 β able to increase the secretion of the enzyme MMP-13 destruction cellular matrix molecules especially type II collagen and decrease the chondrocytes cell synthesize proteoglycans cartilage damage.

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MATERIALS AND METHODS

Animals Model

Female, white, 4-6 months old, weigh around 1500-2500 gram, *New Zealand* rabbits (Oryctologus cuniculuc), were used as an animal model. The rabbits were acquired from Modern Rabbit Farming under Batu Animal Husbandry Department. To accelerate joint cartilage damage OA used incision modeling on the anterior cruciate ligament (ACL). ACL incision procedure refers to a method Hulth modified by the procedure of Balai Besar Pelatihan Peternakan Animal Clinic, Batu, East Java. All care and experimental procedures carried out by the study protocol approved by the Ethics Committee for Health Research, Faculty of Medicine, University of Brawijaya based on the number 372 / EC / KEPK / 09/2016.

Study design

Research design was using experiment. Rabbits divided into six treatment groups, namely Group (1) normal rabbit got the formula DL-methionine 0.25%; group (2) normal rabbit got the formula 0.15% The DL-methionine; group (3) normal rabbit got the formula DL-methionine 0.00%. Then the group (4) rabbit ACTL got the formula DL-methionine 0:25%; group (5) ACTL rabbit gets the formula DL-methionine 0.15% and the group (6) rabbit ACL got the formula DL-methionine 0.0%. Giving formula DL-methionine for 5 weeks and got extra green vegetables as much as 30 g / day / head. Methionine formula feeding conducted in the morning at 07.00 and greens given afternoon at 15.00.

Methionine formula

Low methionine Formula prepared in the Laboratory of Food Technology Nutrition Department of Ministry of Health Polytechnic of Malang. Three formulas methionine is formulated using a mixture of local food with protein, fat and carbohydrates the same, namely 12.86% protein; fat 8.7%, carbohydrates 65.41%. Methionine is used in the form of DL-methionine and the amount added to the formula methionine different (ie 0, 25% DL-methionine (methionine enough)_control formula; 0, 15% DL-methionine (methionine medium) and 0% DL-methionine (lowmethionine). Composition Formula methionine (g / 1000g) as in table 1.

Table 1. Composition of Formula methionine

No	Food ingredients	Formula methionine (per 1000 g)		
		DL-methionine (0.25%)	DL-Methionine (0.15%)	DL-Methionine (0.00%)
1	Corn Starch (g)	600.00	600.00	600.00
2	Bran (g)	100.00	100.00	100.00
3	Polar (g)	100.00	100.00	100.00
4	soy flour (g)	100.00	100.00	100.00
5	Oilseeds (g)	82.0	82.0	82.0
6	Salt (g)	5	5	5
7	Vitamins (g)	2	2	2
7	Mineral (g)	1	1	1
8	DCP (g)	10	10	10
9	DL- Methionine (g)	2.5	1.5	0.00

Blood Collection bunny

Rabbit blood sampling performed on veins *auricular* much as 4*cc,* through a large vein in the ear aseptic event. Then the blood is inserted into *vacutainer* without coagulant (closed warn a red) by 2 cc and *vacutainer* with anti coagulant EDTA (purple cap) of 2 cc.

Body Weight Measurement

Weight measurement rabbit individually performed once a week using electric scale sbrand *CAMCRYACS 15-JC33*type,capacity of 30 kg with a precision of 1 g. Weighing done in the morning before the administration of low methionine formula. Weight gain is calculated by subtracting the results of the weighing of the end of the initial weight expressed in g.

Intake measurement formula methionine

Methionine formula intake was measured by weighing the food remains methionine formula every day for 35 days using scales. *a triple beam* Formula intake of methionine calculated by subtracting the amount of formula given by the number of residual formula is not consumed. The average intake of methionine per day calculated by dividing the total intake of methionine divided 35 day formula and expressed in g per day.

Serum IL-1β measurement

Elisa methode was employed to measure serum IL-1β and performed according to ELISA commercial kit instruction Ray Bio Rabbit IL-1 β, ELL –IL-1β). A 50 µl blanko and standard solution were put pipetted into

empty wells. A total of 50 µl of blood serum each sample and put into the wells and incubated in at 37 °C, covered with thin-foil wrap for 30 minutes. The solution was rinsed 4 times with PBST ,added 50 µl, secondary antibody conjugated with HRP and incubated once more in 37 ° C, covered with thin wrap, for 30 minutes. After that, the solution was rinsed 4 times with PBST and added 50µl tetramethylbenzidine (TMB) was added in a dark room. After 15 minutes of 37 ° C incubation stop solution (in NaOH) was added and the ELISA plate was read at 450 nm wavelength.

Examination of DNA methylation of gene promoters of IL-1β

Based on the analysis of Bioinformatics NCBI, DNA sequencing IL-1β gene is located on chromosome 2 rabbits at the site of the reverse strand and position 97.614851-67.618656 3589-3846 9. The results of DNA sequencing promoter region *geneof IL-1 β* has a length of 285 bp the sequence of the primary design F: TAA GGA GGA ATT GTT GTT TGT TGA T and R: CTC ACT CTT AAT AAA TTT AAA CCC A, Left primer 3589 25 58.94 52.00 5 GATAAGTTGGAATTTGAGTTTGTT, primary Right 3846 25 59.80 44.00 4 AAAACTCTCCTTAATTTTCCCAAAA. Whole-genome DNA isolation results do CT *conversion* by using *the EZ DNA methylation GOLD "Kit* (Catalog Nos.D5005 & D5006) with procedures the protocols listed in the Kit. For PCR mix using KOD-Multi & epidemic Toyobo (catalog F1440K). DNA Sequencing of IL-1 gene is carried out by PCR using Sequence Scanner 2 of Thermo Fisher. Data obtained by equated to see the similarities and differences in the nucleotide sequence of each group and DNA methylation levels of IL-1 β are calculated.

Examination of the expression of IL-1β

Expression of IL-1β in the cartilage tissue of a rabbit knee joints examined Immunohistochemistry with primary antibody rabbit polyclonal anti-IL-1β using a kit *Santa Cruz* (Sc7884). The procedures performed following the protocol of the manufacturer modified, beginning by *blocking endogenous* peroxidase, *blocking* protein un spesifik buffer *(backgroun sniper).* Then the primary anti-rabbit polyclonal anti-rabbit antibody IL-1 β, incubating overnight at 4 ° C. After it was allowed to reach room temperature and washed with BPS 3 x 5 minutes, incubated with secondary antibody *(biotin Conjugate)* for 60 minutes, washed with PBS 3x5 minutes and added to slide with DAB (DAB chromagen: DAB buffer = 1:40), incubated at room temperature for 1-10 minutes and washed with 3x5 aquade minutes. Furthermore added to the slide with solution *Mayer'shematoxilen* (Mayer's *Hematoxilen: Distilled water* = 1: 3) and incubation at room temperature for 1-10 minutes, then Mouting with etellan. Observations chondrocyte cells that express IL-1 β under the microscope O*lympus* B x 51 and 400 times magnification objective.

Level of Cartilage Damage of Knee Joint

Damage to cartilage of the knee joint is assessed from the presence of fissure on the surface of the knee joint cartilage with H & E staining and the proteoglycan content demonstrated from the safranin-O staining intensity. Assessment of cartilage damage of the knee joint with using Mankin's scoring system modification with give score in each sub category, the total of the assessment results of each sub category shows the level of damage of joint cartilage.

RESULTS

Body Weight Animals

Having given formula DL-methionine for 35 days, appeared to be changes in body weight as shown in the chart rabbit weight development rabbit in figure 1

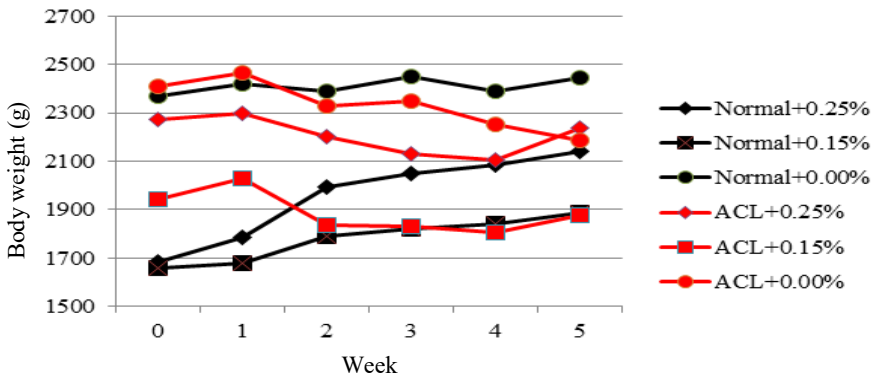

Figure 1. The development of body weight (g/week)

On The first week adaptation phase seemed no weight changes, enters week two begin no weight change. Normal rabbit group body weight tends to rise, the lowest weight gain of about 2.74% in rabbits given formula DL-methionine 0.00%. While the rabbit ACL body weight development tends to fall with the highest weight loss of 7.99% in the rabbit ACL by formula DL-methionine 0.00%. Based on the results test *of one-way ANOVA* against weight gain in normal rabbit group there is a significant difference with $p = 0.024$ ($p \leq 0.05$), and showed no difference with $p = 0677$ ($p > 0.05$) in the group of rabbits ACL. Additions and weight loss associated with the intake of formula rabbit DL-methionine and activity. Figure 2 shows the average intake of food formula DL-methionine per day.

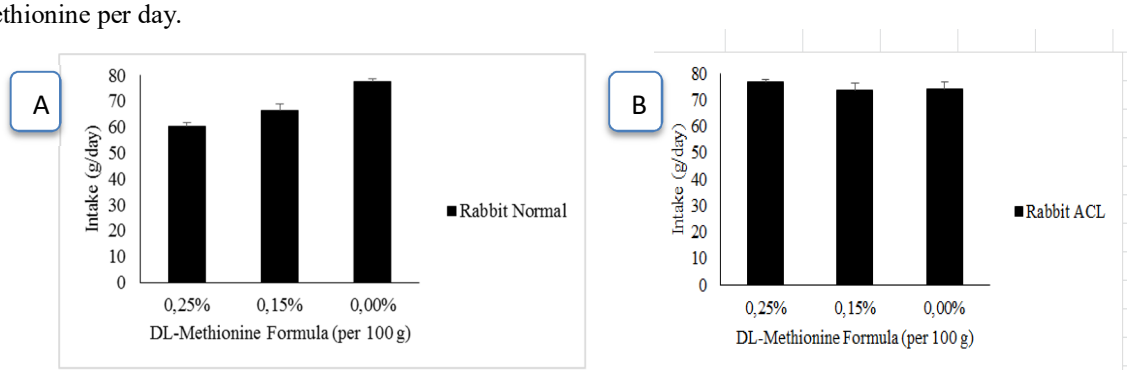

Figure 2. Intake of Food Formula DL-Metionin per day

Serum IL-1β level

Serum IL-1β levels in the normal rabbit group were significantly different between groups with significant values of $p = 0.000$ ($p \leq 0.05$). Administration of a low-methionine formula (DL-methionine 0.00% formula) in normal rabbits had higher levels than rabbits given the formula of DL-methionine 0.25%. In the ACL rabbit group there was a difference in IL-1β levels between rabbit groups with indigo $p = 0.04$ (<0.05). Figure 3. shows the levels of IL-1 beta serum of normal rabbits and ACL rabbits.

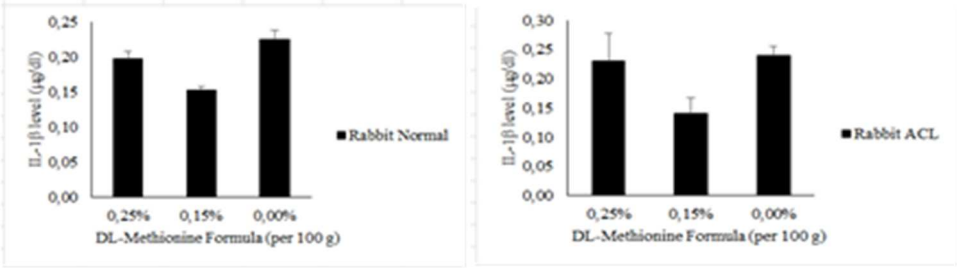

Figure 3. Serum IL-1 level

Methylation DNA gen IL-1β

CpG Based on the analysis of gene IL-1 β CpG in the promoter region *island* (258bp) gene lies only 7 sites IL-1β is the site 25, 48, 81, 116.118, 142, and 153. in normal rabbits and rabbit ACL positioned IL-1β gene in the promoter region of IL-1β gene together, it shows that the incision ACL does not cause changes in methylation patterns IL-1β gene DNA. In the figure 3 shows the position of IL-1β gene in the promoter region of IL-1β gene and gene methylation status of IL-1 β

In rabbits given formula DL-methionine 0.00%, there are 3 sites of gene IL-1β which do not undergo methylation that sites 25, 81 and 153, as indicated by the change in base citosin Guanine (CG) to thymine Guanine (TG) in the DNA sequence in IL_1β gene promoter region. While the group of rabbits given formula DL-methionine contained 0.15% The 25 sites which do not undergo methylation, whereas by the formula DL-methionine 0:25% of seven (7) sites in the gene IL-1β gene promoter region of IL-1 β methylated all. The effect of low intake of methionine on the methylation status as shown in Figure 4.

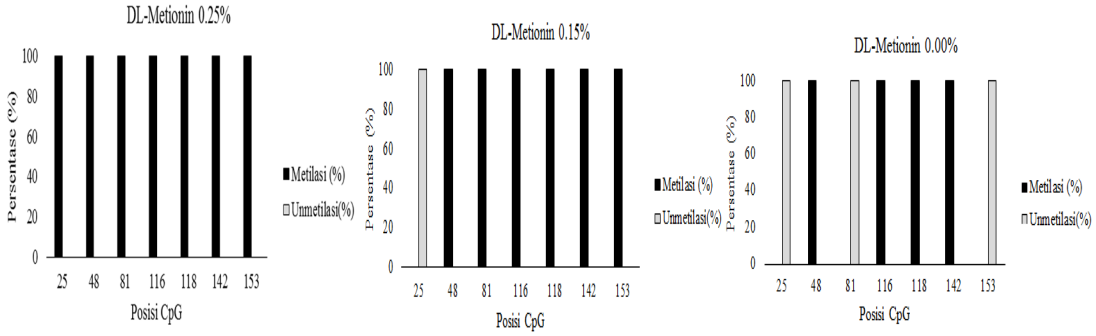

Figure 4. A low intake of methionine induces a decrease in IL-1 β rabbit gene methylation

Figure 4, shows that a low methionine formula (Dl-methionine 0.00%) has an effect decrease IL-1β gene methylation or increase unmetilation of IL-1β gene, thus enhancing IL-1β expression. Kruskal Wallis test showed that there was significant difference $p \leq 0.005$ to IL-1 β methylation between rabbit groups

Expression of IL-1 β in Cartilage Knee Joints

Interleukin -1 β (IL-1β) is a local inflammatory mediators have an important role in the destruction of joint cartilage. Based on the calculation of the average expression of IL-1 β in the knee joint cartilage of rabbits are presented in figure 5

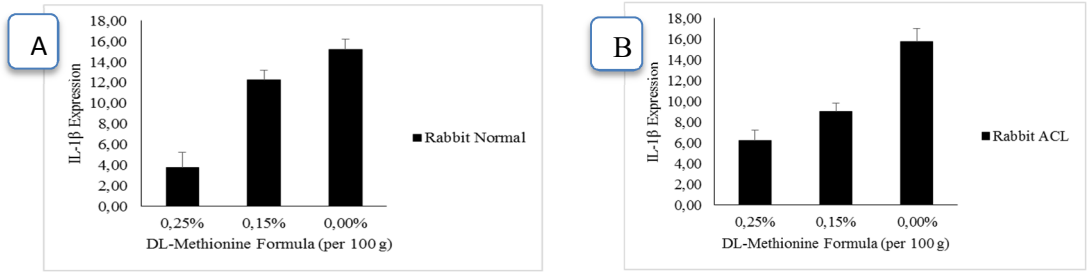

Figure 5. IL-1β expression in cartilage joints of the knee joint (A) normal rabbit group, (B) group of ACL rabbits

The results of the one-way ANOVA test on the expression of IL-1β in normal rabbit group significant difference with significant p value $\leq 0.05 = 0.01$. In the rabbit ACL group of one-way ANOVA test results, there were significant differences with significant value ≤ 0.05 $p = 0.00$. Results of examination with immunohistokima expression of IL-1β in cartilage tissue in figure 6.

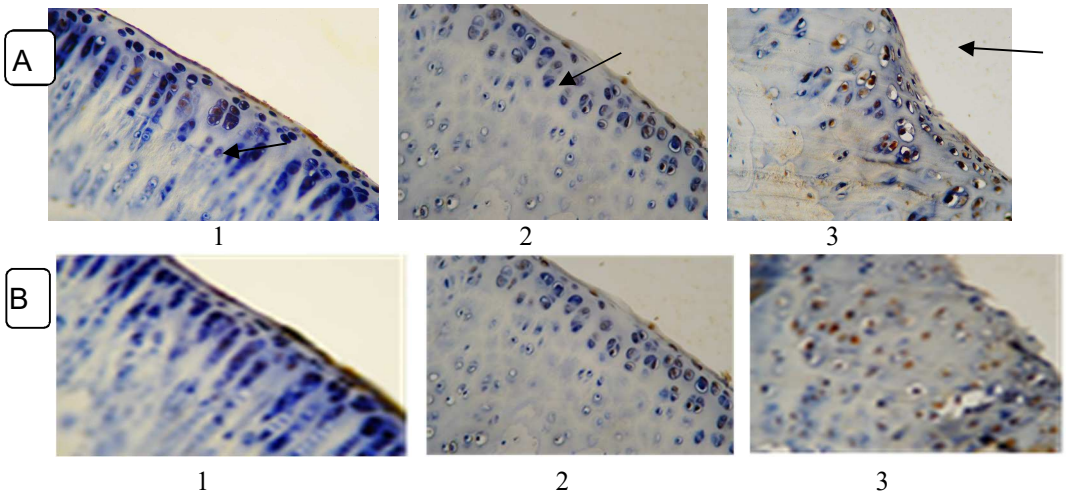

Figure 6. Expression of IL-1β by immunohistochemical methods (magnification 400 x) looks brown in the cytoplasm of cells.

Description:
A. The preparation of joint cartilage normal rabbit knee joint cartilage
B. Preparations knee incision ACL
1. Rabbit + Formula DL-methionine 0.25%
2. Rabbit + Formula DL-methionine 0.15%
3. Rabbit + Formula DL -metionin 0.00%

Cartilage Damage knee joint

Total average score of cartilage damage assessment of the rabbit knee joint as presented in figure 7

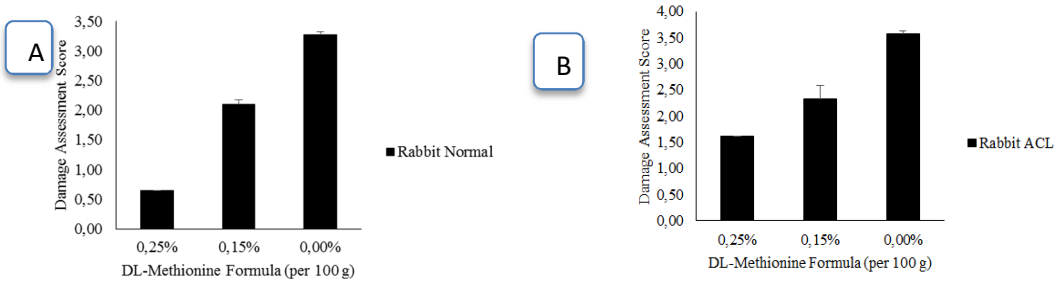

Figure 7. Score of of cartilage damage (A) normal rabbit group, (B) group of ACL rabbits

Based on the test *Kruskal-Wallis* showed that the obtained value of p = 0.006 less than 0.05, it can be concluded least there were no differences between groups, followed by test. Histology of cartilage damage based on changes in the structure of cartilage that rated the fissures in the surface layer joints with H & E staining and staining with safrani-0 to evacuate glycosaminoglycan content judged from the intensity of the illumination. Histology of cartilage structure as shown in Figure 8 and picture lighting intensity as in Figure 9.

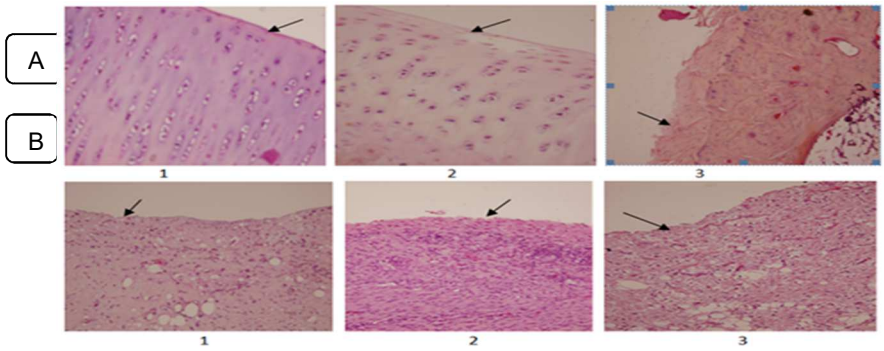

Figure 8. Histology of the rabbit knee joint cartilage

Description:
A. The preparation of joint cartilage normal rabbit knee joint cartilage
B. Preparations knee incision ACL
1. Rabbit + Formula DL-metionin 0.25%
2. Rabbit + Formula DL-metionin 0.15%
3. Rabbit + Formula DL- methionine 0.00%

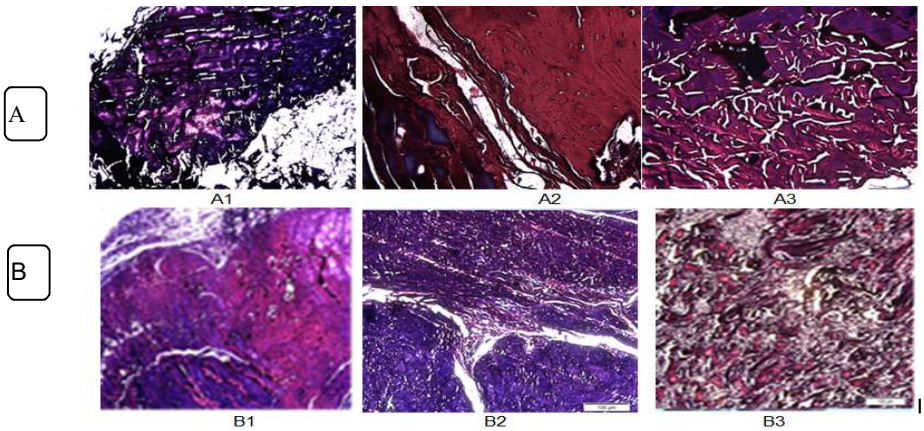

Figure 9. Safranin O staining intensity in joint cartilage

Description:
A. The preparation of joint cartilage normal rabbit knee joint cartilage B. Preparations knee incision ACL
1. Group by giving formula 0:25% DL- methionine (control)
2. Group with a formula giving methionine DL-methionine 0.15% the
group with the provision of the formula 3. methionine 0.00% DL-methionine

DISCUSSION

In this study the formula giving low methionine (DL-methionine 0.00%) in normal rabbit 35 days can increase the weight of about 65.0 g (2.74%) was lower than in rabbits given formula DL- methionine 0.25% reaching 375 g. Similar results were observed in mice fed a diet low in methionine (DL-methionine 0.00%) during the 33-day weight gain is lower than the control group to 38% [21]. Other studies have diets low in methionine administration in rats for 3 months of weight gain of 45% was slightly lower than the control group [7]. Methionine restriction in mice can reduce fat mass, improve insulin sensitivity and affect fat metabolism, energy and health status [14, 15, 20]. In normal conditions the restriction of methionine to maintain body weight remains constant by increasing the use of energy, involving an increase in energy expenditure [13].

Levels of Interleukin-Iβ serum

In this study, administration of DL-methionine formula different in normal rabbits causes a difference to the levels of serum IL-1β was significantly ($p = 0.008$) and no difference ($p = 0.064$) in the group of rabbits ACL. Giving formula DL-methionine 0.25% in normal rabbit group is able to inhibit the increase of serum IL-1β and giving formula DL-methionine 0.00% may increase serum levels of IL-1β and thus potentially have an effect on low-level inflammation.

Interleukin -1 (IL-1β) is a major inflammatory cytokine that plays an important role in the infection process and aging-related diseases as well as the innate immune response. In older people IL-1β play a role in normal homeostasis and as an inflammatory response is responsible for the development of chronic disease [6]. As a major inflammatory cytokines interleukin -1 β (IL-1β) have autocrine and paracrine effect, so that the serum levels of IL-1β is often used as an index of non-specific inflammation. Physiology of food intake involved in the inflammatory response and oxidative stress [10], changes in plasma amino acid concentrations causing malnutrition and inflammatory conditions

DNA Methylation

DNA methylation becomes one of the epigenetic modifications which is the reaction of adding methyl groups (CH3) to the carbon position of 5 'cytosine and producing the silencing gene phenotype.This addition of methyl (CH 3) groups in C causes chemical structure changes that can not be attached by transcription factors binding to the promoter's DNA causing the inactivation of genes (silencing genes). This DNA methylation process is mediated by the DNMT 1 enzyme and DNMT1 activity is affected by the level of S-adenosylmethionine (SAM) [4]. Methionine as an essential amino acid derived from food can contribute about 60% of exogenous methylsatures to S-adenosylmethionine (SAM) substrates and important DNMT1 enzyme activity in the methylation process.

In this research, the provision of DL-methionine 0.00% causes the availability of SAM methyl (CH3) endogenous sources and the activity of DNMT decreases. In this study, rabbits who were given DL-methionine 0.00% formula contained 3 un-methylated CpG IL-1β sites.Several studies that have been done on OA patients show that there is a loss of DNA methylation in the CpG IL-1β promoter region that will express the IL-1β gene [22]. DNA demetilation in the long-acting IL-1β gene promoter region causes a deviation of persistent gene expression [11,12,16].

Expression -1β

Under normal conditions IL-1β plays a role in maintaining homeostasis and as an inflammatory response responsible for the development of chronic disease that occurs in the elderly [6]. Bonds between IL-1β receptors and IL-1β ligand are able to alter condyting phenotypes to more catabolic condrosites [2]. In this study normal rabbits were given a low-methionine formulation (DL-methionine 0.00%) having a 5-fold higher-than-normal 5-fold IL-1β expression compared to a normal rabbit given a 0.25% DL-methionine formula.

In ACL rabbits IL-1β gene expression is higher than normal rabbit, but has a DNA methylation status of IL-1β gene same as normal rabbit.Several previous studies have shown that ACL incision in rabbits causes condylits to synthesize IL-1β at higher levels in post-trauma [6,11]. Other studies have shown that acute ACAR acute incarceration in experimental animals leads to biochemical, biomedical changes and provides a higher release of inflammatory mediators [1,6,10]. In a study using human subjects, after a knee injury occurs a metabolic imbalance and biomechanical changes that aggravate cartilage damage and cause osteoarthritis [2,4].

Cartilage Damage Knee Joint

In this study a low intake of methionine (a formula DL-metionin 0.00%) can be increases the risk of cartilage damage of the knee joint through decreased methylation of the Il-1β gene DNA. A low intake of methionine causes the availability of methionine in the body is reduced resulting in a decrease in the formation of S-adenosylmethionin (SAM) and the activity of the enzyme DNMT1.

Decreased DNA methylation of the IL-1β gene induces expressed IL-1β which is a major inflammatory mediator in cartilage damage. Increased expression of IL-1β in the joint cartilage tissue causes cell chondrocytes produce MMP-13 high and inhibit proteoglycan synthesis. MMP-13 is a degrading enzyme extracellular components matrix joint cartilage especially collagen type II. In this study increased intake of methionine can inhibits decreased DNA methylation of IL-1β gene, all CpG sites of the IL-1β gene on the promoter region of the IL-1β-termylated and IL-1β genes were low in the rabbit group given the DL-methionine 0.25% formula [23,24].

CONCLUSION

This study showed that a low intake of methionine (DL-methionine 0.00%) may cause low-level inflammation, and increase joint cartilage damage through decreased DNA methylation of IL-1β gene, increased IL-1 β expression in joint cartilage tissue . Further research is needed to determine the effect of low intake of methionine on the expression of TGF-β as an anabolic gene cytokine in cartilage tissue.

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