

Liver metabolic activities of Pasundan cattle induced by irradiated chitosan

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Abstract. *Mushawwir A, Arifin J, Darwis D, Puspitasari T, Pengerteni DS, Nuryanthi N, Perman R. 2020. Liver metabolic activities of Pasundan cattle induced by irradiated chitosan. Biodiversitas 21: 5571-5578.* A total of one hundred and twenty-five, 2-3 year old male Pasundan cattle were used as livestock samples during the three months of this research. They were selected from the local cattle breeding and development center in Ciamis. The animal samples were randomly allocated to 5 treatment groups. One group served as the control, or without irradiated chitosan, while the others were used as treatment in varying levels. Each treatment group involved five replicates with 25 Pasundan bulls per treatment i.e five Pasundan bulls per replication. Each group was provided with the following rations: C0 = Control group, without IC (0 ppm IC); C1 = 350 ppm Irradiated Chitosan (IC); C2 = 400 ppm IC; C3 = 450 ppm IC; and C4 = 500 ppm IC. Irradiated chitosan was obtained through the following steps: extraction, deacetylation, and irradiation of chitin using gamma rays. Five mL of blood samples were collected from each bull at the beginning of each month of this experiment, which totaled three months. The blood samples were sucked from the tail/coccygeal vein using a sterilized syringe and vacuum tube containing K3EDTA. The plasma was used to determine the concentration of parameters related to liver metabolism through an automatic biochemical analyzer Kenza 240TX model from Biolabo, using a commercial kit. Each procedure was followed based on the Biolabo kit (Franch) and Randox kit (UK). This study showed that IC reduces the activity of glycogenolysis and glycolysis, but is accompanied by improvements in the biochemical conditions of liver cells. This is a favorable condition for the metabolism of Pasundan bulls in order to enhance their growth and reproduction.

Keywords: Cattle, irradiated chitosan, liver, metabolic

INTRODUCTION

Pasundan cattle (or Rancah cattle) are one of the local ruminants that can be reared in Indonesia. These cattle are a cross of *Bos javanicus* x *Bos indicus*. These are local cows that have been growing in West Java for a long time, especially in areas with low topography. From the climatological aspect, they can adapt to Indonesian tropical climates (Sutarno and Setyawan 2015, 2016). However, Pasundan cattle appear to have many deficiencies in terms of performance, i.e. growth and reproduction, when compared to local crossbred cattle. Aamir et al. (2010) and Adrial (2010), observed that local cattle in Indonesia do not show high growth. The Pasundan cow performance was published by the Department of Animal Husbandry in 2013, and its reproductive traits include: First calving at 30-40 months, sex maturity at 25-30 months, gestation length 8.5-10.0 months, first estrus 18-24 months, estrus periods (18-24 days) and calving interval (1.1-1.3 years). The carcass traits of Pasundan cattle such as water holding capacity ranges between 23-30%, cooking loss 25-45%, tenderness 35-96 mm/10s/g. The slaughter weight, hot carcass, and dressing percentage of Pasundan bull weights

are 240.40 kg, 127.20 kg, and 53.02%, respectively (Aamir et al. 2010; Department of Animal Husbandry, 2013; Nugraha et al. 2016).

Researchers have discovered various strategies for improving the performance of cattle. Abdullah et al. (2007), stated that crossbreeding techniques can improve the genetic quality of livestock, and the reproductive system is enhanced by inducing the performance of their hormonal system (Khan et al. 2015). However, no report of metabolic induction studies has as a good strategy for Pasundan cattle. The induction of metabolism using natural extracts is the first step in the application of nutrigenomics for ruminant development.

The natural extracts added to the feed showed positive results for increased metabolism, which was related to changes in gene expression within cells. He et al. (2019) demonstrated the effectiveness of garlic extract on gene expression in increasing immunity and performance of experimental animals. The same results were obtained by Tian et al. (2015, 2016); Adriani et al. (2015); Kusmayadi et al. (2018); Mushawwir et al. (2018); Soisuwon and Chauyuchwing (2013) and Kamil et al. (2020), that plant extract improves the performance of nutrient absorption

and metabolic rate in the liver and also increases livestock growth.

Chitosan is one of the most widely used natural extract ingredients. It originates from the exoskeleton of arthropods such as crabs, shrimp, insects, and other species in the crustacean family (Bueter et al. 2014; Carrol et al. 2016). It possesses multifunctional properties, including antibacterial (Cai et al. 2014; Allen et al. 2015; Moreno et al. 2019) and antioxidant characteristics (Yarrus et al. 2012; Qiu et al. 2018; Araujo et al. 2015). Chitosan is a linear polysaccharide in form of β -(1,4)-2-amino-2-deoxy-D-glucopyranose, where its structure is similar to glycosaminoglycans. Furthermore, it is a hetero-polymer of glucosamine (2-amino-2-deoxy- β -D-glucose) which binds to β -1,4 polymer and contains N-acetyl-glucosamine. New techniques for chitosan processing are continuously developed, and one of these (Darwis et al. 2014) is irradiated chitosan. There are no previous research on the application of irradiated chitosan in animals, especially cattle.

Irradiated chitosan (IC) produced through the application of nuclear technology, has better quality due to the shorter nature of the glucosamine polymer, therefore the molecular weight is lighter. This irradiated chitosan is absorbed into livestock tissue cells to interact with the biochemical metabolic system in the liver. This study aims to determine the effect of irradiated chitosan on the metabolism of Pasundan bull liver cells.

MATERIALS AND METHODS

Study area

This research was carried out in Ciamis District, West Java, which has become a center for the development and breeding of Pasundan cattle in the West Answer Province, Indonesia. This was the main reason for selecting this location, in order to facilitate the use of Pasundan cattle as research samples. Moreover, homogeneous samples can be determined according to their biological aspects. The research location is shown in Figure 1.



Figure 1. Research location of Pasundan cattle in Ciamis District, West Java, Indonesia. A. Male, B. Female of Pasundan cattle

Experimental design

Animal samples and treatment

A total of one hundred and twenty-five, 2-3 year old male Pasundan cattle were used as livestock samples for the three months of this research. They were selected from the local cattle breeding and development center in Ciamis. The animal samples were randomly allocated to 5 treatment groups and placed in the colony pen, measuring 4x3 m. Each pen was allocated to a single group. In this study, 25 colony cages were used in an open house with a tail to tail system. The average housing temperature was 25-32⁰C, while the relative humidity was maintained between 74-80% during the experimental period,. The animals were provided with water and food ad libitum.

The animal samples were randomly allocated to 5 treatment groups: control or without IC, and the other group as a treatment group at various IC levels. Each treatment group involved five Pasundan bulls per replication. The rations and treatment provided include the following: (i) C0: Control group, without IC (0 ppm IC), (ii) C1: 350 ppm Irradiated Chitosan (IC), (iii) C2: 400 ppm IC, (iv) C3: 450 ppm IC, (v) C4: 500 ppm IC.

Preparation of Chitosan Irradiation (IC)

Irradiated chitosan was obtained through the following procedure:

Extraction of chitin

The extraction of chitin from shrimp shells includes two stages, namely: deproteination and demineralization. These involved the removal of protein using 1 N NaOH solution at room temperature for 24 hours. Afterward, the shell was rinsed with water to a neutral pH. The deproteinated shell was then suspended in 1 N hydrochloric acid solution (HCl) at room temperature for 24 hours to remove mainly minerals calcium carbonate. The residue collected and washed to neutral in tap water. Lastly, it was dried in the sun to obtain chitin.

Chitin deacetylation

Before the deacetylation process, a mixer was used to cut the chitin into 1 mm flakes. Deacetylation of chitin was carried out under alkaline conditions using 50% NaOH solution at 90°C. Afterward, the chitosan was collected and washed using hot water and soaked in distilled water for 1 hour to obtain a neutral pH.

Irradiation

Low molecular weight chitosan was produced by irradiating its flakes using Co⁶⁰ gamma rays at a dose rate of 10 kGy/hour.

The technique of treatment and basal ration

Irradiated chitosan (IC) was dissolved in 1 L of water, which obtained doses or concentrations of 350, 400, 450, and 500 ppm. Afterward, each experimental animal was fed 2 kg of concentrate according to the treatment group. This IC + concentrate was administered daily before the provision of drinking water and forage. The concentrate composition is shown in Table 1.

Sample collection and analysis

Five mL of blood samples were collected from each Pasundan bulls at the beginning of each month. They were sucked from the coccygeal vein using a sterilized syringe and vacuum tube containing K3EDTA. Slide alcohol 70% was applied on the medial side of the tail before and after blood collection to prevent pathogenic infection.

The blood samples collected were also centrifuged to separate the plasma. The plasma was used to determine the concentration of parameters related to liver metabolism via an automatic biochemical analyzer from Biolabo KENZA 240TX model, using a commercial kit. Each procedure of the analysis was followed based on the Biolabo kit (French) and Randox kit (UK). The experimental protocol and the animal samples followed good animal practice. Furthermore, the authors declare that there are no competing interests.

Data analysis

The one-way analysis of variance (ANOVA) was used to analyze the liver metabolic levels. The SPSS software Version IBM 21 was used to obtain a completely randomized design. The difference between the mean of all treatments was obtained using Duncan's multiple range tests (SPSS IBM, 2010) at a 95% significance level or $\alpha = 5\%$.

Table 1. Composition of nutrient concentrates used during the study

Nutrients*	(%)
Carbohydrate	12
Dry matter	88
Crude protein	18
True digestibility nutrient	75
Crude fat	7
Crude fiber	7
Neutral detergent fiber	30
Calcium	0.7
Phosphorus	0.6

Note: *Local Cattle Breeding Centre, Ciamis (2019).

RESULTS AND DISCUSSION

Glycogenolysis pathway

The effect of various levels of IC on the concentration of glycogenolysis pathway metabolites in the Pasundan bulls is shown in Table 2.

In this experiment, Pasundan bulls were housed in cages with and without IC treatment. The average glycogen level in the sample animals without IC was 0.97 mg/g (Table 2). The increase in glycogen levels in the IC-treated Pasundan bulls group was significant ($P < 0.05$), starting with IC levels of 450 and 500 ppm, namely 1.32 and 1.48, respectively. These results indicate that during treatment, a minimum of 450 ppm causes a decrease in glycogen breakdown in liver cells. Meanwhile, glycogen decrease in the group without IC treatment caused the degradation or catabolism of glycogen (glycogenolysis). This catabolism also occurred in the Pasundan bulls group treated with 400 ppm IC.

The effectiveness of IC was observed in the group that was administered with 450 and 500 ppm IC. This was shown by the glycogen levels in the livestock sample group, which was significantly ($P < 0.05$) higher than the cattle group without IC treatment, and with IC treatment at 350 and 400 ppm. The results of this study indicate that IC administration reduces the rate of energy supply from alternative pathways, such as glycogenolysis (Mushawwir et al. 2010, 2011; Slimen et al. 2016). Previous research has also shown that natural extracts reduce glycogenolysis in ruminants (Aziz et al. 2012; Ao et al. 2020; Paiva et al. 2016; Fabris et al. 2017; Valle et al. 2017), and decreases glucagon activity (Soisuwon et al. 2013; Adrian and Mushawwir 2020; Kamil et al. 2020).

The intermediate metabolites such as glucose 1-phosphate, glucose 6-phosphate, and the enzymes involved, including glycogen phosphorylase, phosphoglucomutase, and glucose 6-phosphatase, during glycogenolysis, appeared to be increased in the livestock group not given IC. Increasing the level of these intermediates occurs due to an increase in glycogenolysis. Previous research also showed an increased profile of intermediate metabolites with the same profile (Tanuwiria et al. 2011; Gray et al. 2015; Mingoti et al. 2016; Siskos et al. 2017).

The increased levels of intermediates and enzymes involved in the glycogenolysis pathway occurred due to the increased rate of glycogen catabolism. The group of livestock samples that were not given IC showed a significant increase in intermediate metabolites ($P < 0.05$). In Table 2, it is observed that the level of the intermediate compound glucose 1-phosphate is 0.42 IU/dL, was significantly higher ($P < 0.05$) in the livestock group given 500 ppm IC, namely 0.20 IU/dL, likewise, for the intermediate compound glucose 6-phosphate 0.31 (without IC) to 0.22 IU/dL (500 ppm IC). The same results also occurred for the catalytic enzymes of glycogenolysis. The results of previous studies showed an increase in the levels of the enzyme catalyst for the glycogenolysis pathway with increased glycogen biocatalyst activity (Holt et al. 2010; Odore et al. 2011; Monteiro et al. 2016). The administration of natural extracts inhibited the action of the

enzyme catalyst glycogenolysis. This was also discovered by Adrini et al. (2015), Singh and Gupta (2015); Mahmoud et al. (2018); He et al. (2019).

Overall, the effectiveness of IC in reducing the rate of glycogenolysis shows that it is able to improve metabolic balance (Antosiewicz et al. 2006; Peinado et al. 2012) related to energy regulation and homeostasis (Roland et al. 2016). From the results of this study, metabolic balance and the achievement of homeostasis with or without IC administration causes no significant difference in glucose levels between treatment groups. Therefore, this shows that glucose is an important buffer for body fluids (Picker et al. 2013; Lee et al. 2014; Loyau et al. 2014), and maintains blood viscosity (Renaudeau et al. 2012; Ma et al. 2014; Roland et al. al 2016; Mushawwir et al. 2018), as well as the osmotic pressure of dissolving cells (Mushawwir et al. 2011; Royer et al. 2016; Hernawan et al. 2017).

Glycolysis pathway

The effect of various levels of IC on the metabolite concentrations of the glycolysis pathway in the Pasundan bulls is shown in Table 3.

Glycolysis is a biochemical pathway to degrade glucose into simpler compounds, namely pyruvic, acetyl co-A, and citric acid. This pathway is essential for providing energy precursors. However, the main route for the provision of these energy precursors in ruminants is gluconeogenesis.

The results of this study are expected to comprehensively explain the biochemical phenomena that occur in supplying energy for Pasundan bulls. Therefore, the results show that the rate of glycolysis is highest in the Pasundan bulls group without chitosan administration, as shown in Table 3. This conclusion is made due to the high

glucose breakdown products in this group. In table 3, the lactate levels in the Pasundan bulls group without IC administration were significantly higher ($P < 0.05$), precisely 26.89 mg/dL, compared to Pasundan bulls with 400-500 ppm administration. The same phenomenon was also observed in the metabolite D-glyceraldehyde 3-P, including the activity of the glucose 6-P Dehydrogenase enzyme.

This is similar to the results in Table 1, where the glucose levels of Pasundan bulls were not significantly different ($P > 0.05$) in all treatment groups. Therefore, an interesting result in Table 2 is an increase in pyruvic acid levels with increasing levels of IC administration. Their pyruvic level at 500 ppm IC was the highest ($P < 0.05$), precisely 25.76 mg/dL among all treatment groups. The same phenomenon was observed in the citric acid profile. Therefore, it can be concluded that the increase in pyruvic and citric acid levels is not accompanied by an increase in glucose levels. Furthermore, pyruvic and citric acid levels are not dependent on glycolysis. These results strengthen the theory that IC is able to stimulate an increase in gluconeogenesis. Researchers have previously observed increasing levels of pyruvic and citric acid with rising rates of gluconeogenesis (Silver et al. 2012; Pickler et al. 2013; Cayan and Erener 2015; Eyng et al. 2015). The investigations by Cai et al. (2014) showed that there is an increase in lipolysis through the gluconeogenesis pathway which influences the pyruvic acid increase in plasma. Similar results were observed by Tapola et al. (2008), that the rise in pyruvic acid due to chitosan occurs with the degradation of cholesterol, forming acetyl Co-A and pyruvic.

Table 2. Liver metabolic by glycogenolysis pathway in Pasundan cattle without and with fed Irradiated Chitosan (IC)

Metabolites	0 ppm IC	IC (ppm)			
		350	400	450	500
Glycogen (mg/dL)	0.97±0.01 ^a	1.13±0.16 ^a	1.16±0.12 ^a	1.32±0.21 ^b	1.48±0.13 ^c
Glycogen phosphorylase (IU/dL)	0.43±0.01 ^a	0.35±0.02 ^{ab}	0.32±0.02 ^b	0.30±0.01 ^{bc}	0.26±0.01 ^c
Glucose 1-phosphate (IU/dL)	0.42±0.01 ^a	0.37±0.01 ^b	0.32±0.01 ^b	0.35±0.02 ^c	0.21±0.01 ^d
Phosphoglucomutase (IU/dL)	0.47±0.01 ^a	0.43±0.03 ^a	0.38±0.02 ^b	0.33±0.02 ^c	0.24±0.02 ^d
Glucose 6-phosphate (mg/dL)	0.34±0.02 ^a	0.27±0.02 ^a	0.31±0.03 ^a	0.23±0.01 ^b	0.20±0.01 ^b
Glucose 6-phosphate (IU/dL)	0.31±0.01 ^a	0.31±0.01 ^a	0.34±0.02 ^a	0.34±0.02 ^b	0.22±0.01 ^b
Glucose (mg/dL)	73.53±2.53 ^a	72.95±3.21 ^a	72.82±3.05 ^a	71.92±2.27 ^a	72.76±2.03 ^a

Note: Values are means±Standard Error; IC: Irradiated Chitosan, ^{a,b} Averages within a row having different superscripts are significantly different ($p < 0.05$)

Table 3. Liver metabolite levels during the glycolysis pathway in Pasundan cattle with and without Irradiated Chitosan (IC)

Metabolite	0 ppm IC	IC (ppm)			
		350	400	450	500
Pyruvic acid (mg/dL)	19.83±1.12 ^a	20.04±1.09 ^a	21.72±1.07 ^{ab}	23.64±1.03 ^b	25.76±2.06 ^c
Lactate (mg/dL)	26.89±1.07 ^a	25.79±2.05 ^a	21.04±1.05 ^b	18.15±1.05 ^c	15.91±1.05 ^d
D-Glyceraldehyde 3-P (IU/dL)	2.83±0.03 ^a	2.76±0.01 ^a	2.02±0.01 ^b	1.77±0.02 ^c	1.54±0.03 ^c
Glucose 6-P Dehydrogenase (IU/dL)	2.86±0.12 ^a	2.25±0.13 ^a	1.79±0.11 ^{ab}	1.33±0.12 ^b	1.23±0.11 ^b
Citric Acid	1.11±0.07 ^a	1.14±0.10 ^a	1.46±0.10 ^a	2.06±0.10 ^b	2.87±0.06 ^c

Note: Values are means±Standard Error; IC: Irradiated Chitosan, ^{a,b} Averages within a row with different superscripts are significantly different ($p < 0.05$)

Table 4. Plasma Biochemistry Related Liver Metabolism in Pasundan cattle with and without Irradiated Chitosan (IC)

Plasma biochemistry	0 ppm IC	IC (ppm)			
		350	400	450	500
Glutamate oxaloacetate transaminase/GOT (IU/dL)	61.68±2.14 ^a	60.37±3.06 ^a	57.83±2.01 ^b	56.66±3.01 ^b	52.26±2.02 ^c
Glutamate pyruvate transaminase/GPT (IU/dL)	57.78±1.24 ^a	54.53±1.63 ^b	52.78±1.05 ^c	49.31±1.13 ^d	49.85±1.27 ^e
δ-Glutaryl transferase/δGT) (IU/dL)	42.26±2.13 ^a	42.04±2.34 ^a	38.52±2.18 ^b	37.26±2.52 ^b	35.95±2.31 ^c
Creatine (mg/dL)	42.52±2.51 ^a	41.39±2.25 ^a	39.24±2.38 ^b	36.18±2.29 ^c	33.85±2.61 ^d
Creatine kinase/CK (mg/dL)	31.09±3.31 ^a	29.93±3.04 ^a	27.27±2.12 ^b	26.84±2.24 ^b	25.74±2.15 ^c

Note: ¹ Values are means±Standard Error, ^{a,b} Averages within a row having different superscripts are significantly different (p<0.05); IC: Irradiated Chitosan

Plasma biochemistry

The effect of various levels of IC on the plasma biochemistry concentrations related to liver metabolism in Pasundan bulls is shown in Table 4. The results in Table 4 show that the levels of the transaminase enzymes, GT, creatine, and creatine kinase were significantly higher (P <0.05) in the group without IC administration, compared to the treatment groups. Therefore, IC is very effective in reducing the levels of these compounds.

The liver is one of the organs most affected by metabolic activity. One of the natural mechanisms of the liver is damaged to its cells due to free radicals of metabolic activity, which causes liver damage in livestock. Free radicals in liver cells result in the failure of carbohydrate metabolism, fat, and protein synthesis, ultimately resulting in the death of liver cells and tissue (Dhanasekaran et al. 2011; Vizzotto et al. 2015). Furthermore, the negative impact of high metabolic rate is oxidative stress, indicated by increased fat peroxidation and decreased enzymatic and non-enzymatic antioxidants (Ippolito et al. 2014; Xu et al. 2015;).

Damage to liver cells is indicated by damage to their cell membrane, which leads to cell death. This results in the migration of liver enzymes into the vascular system. Consequently, the levels of the enzyme transaminase, both SGOT and SGPT increase significantly (P <0.05), which occurred in the blood plasma of the Pasundan bulls group without IC administration.

The metabolic activity has an impact on liver cells, and causes oxidative damage to lipids and lipoproteins, which are components of cells (Tanuwiria, 2011; Roland et al. 2016; Vizzoto et al. 2015). Several studies have shown that oxidative damage causes the emergence of free radicals, which in turn, damages lipid structure (Tian et al. 2016; Damaziak et al. 2017; Hernawan et al. 2017), enzymes (Burdick et al. 2011), nucleic acids and proteins (Carrol et al. 2016) as well as cell and DNA damage (Cai et al. 2014; Shin et al. 2010; Holt et al. 2019).

IC is effective in reducing or overcoming cell death, both as a direct effect of aflatoxins, as well as an indirect impact, especially in increasing free radicals that cause cell death. The effectiveness of irradiated chitosan in preventing damage to liver cells is shown to be optimum at 500 ppm IC, followed by a significant decrease (P<0.05) in AST levels of 52.26 and SGPT 49.85, as shown in Table 4. SGOT and SGPT blood plasma in the Pasundan bulls group decreased at 350-450 ppm IC administration,

including the group without IC administration. Therefore, the migration of SGOT and SGPT into the blood vessel system due to damage of liver cells decreased significantly.

The results of this study prove that irradiated chitosan with a molecular weight of 30-50 kD successfully interacted and generated molecular signals in cells. In addition to signals that stimulate cell repair, irradiated chitosan can counteract the negative impact of free radicals, especially from reactive oxygen species (ROS).

Previous researchers have reported the biochemical mechanism of chitosan in preventing, overcoming, and repairing liver cells. Carroll et al. (2016) and He et al. (2019) demonstrated its ability to prevent ROS signaling through the activation of Cyclic Guanine Monophosphat-Adenyn Monophosphat Synthase (cGAS) and Stimulator of Interferon Genes (STING) in dendrite cells. Activation of cGAS-STING consequently induces the activation of interferon dependent type-1 (I IFN-dependent), thereby enabling the dendrite cells to become active. Furthermore, Blaauboer et al. (2015) and Cai et al. (2014) reported that the activation of dendrite cells increased the polarization response of T-helper 1 (TH1) cells protein. Th1 activation increases gamma interferon (IFN g), which stimulates immunoglobulin G (IgG) in the cytoplasm. In addition, enriched signal pathways help to prevent cell damage (Bueter et al. 2014).

The impact of IC administration in preventing cellular damage is shown by the ability of chitosan to activate ATP realists (Gehrke et al. 2013), which stimulate the activation of nuclear protein receptors 3 (NLRP3 inflammasome) (Lin et al. 2014). The signal from NLRP3 stimulates the expression of the interleukin-1β (IL-1β) and interferon-gamma (IFNγ) genes. Increased levels of interleukin-1β (IL-1β) and interferon-gamma (IFNγ) prevent ROS from inhibiting Cyclic Guanine Monophosphat-Adenyn Monophosphat Synthase (cGAS) and Stimulator of Interferon Genes (STING) (Dubensky et al. 2013; Ippolito et al. 2014; Tanuwiria et al. 2020). Therefore, the damage to liver cells is effectively treated with IC.

This study shows that IC reduces the activity of glycogenolysis and glycolysis, but is accompanied by improvements in the biochemical conditions of liver cells. This is favorable for the metabolism of Pasundan bulls which support the achievement of higher performance (growth and reproduction).

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