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EFFECTS OF FEEDING A1 AND A2 COW MILK-BASED DIET ON BIOCHEMICAL PARAMETERS IN DIABETIC MICE MODEL

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Abstract– Streptozotocin is naturally occurring target specific alkylating agent derived from gram-positive bacterium *Streptomyces achromogenes* species that triggers an inflammatory mechanism leading to preferential destruction of pancreatic beta cells which is responsible for hyperglycemic states in experimental C-57 BL/6 mice. Cytotoxic effect of Streptozotocin is well established to build type 1 diabetes mellitus (T1DM) study model. We induced diabetes in C57 bl/6 mice by injecting Streptozotocin (dissolved in 0.1 M sodium citrate buffer at pH of 4.4) intraperitoneally at dose of 45mg per kg of body weight for consecutive 5 days. The mice were fed with A1A1 and A2A2 milk and their response was seen at 3 months with respect to their controls. A2A2 diet was not related to the induction of biochemical abnormalities while A1A1 and A1A2 diet have some association to cause some inflammatory reactions.

INTRODUCTION

Milk is a highly complex biological fluid that contains various components such as proteins, lactose, fats, vitamins, and minerals. One of the milk proteins, called bioactive peptides, is known for its biological active properties. Studies have shown that increased milk consumption, as a result of altered dietary habits, has been associated with an increased incidence of type I diabetes. This form of diabetes is characterized by a complete lack of insulin and subsequent hyperglycaemia due to the immune T-cell-mediated attack and destruction of the pancreatic insulin-producing b-cells. This type of diabetes is commonly known as insulin-dependent diabetes (IDDM) because it requires insulin injections for management. Interestingly, there is a contradictory relationship between milk consumption and both type I and type II diabetes. Research has demonstrated the diabetogenic effects of milk protein, which can lead to obesity, hyperinsulinemia, and altered glucose homeostasis. It has been suggested that environmental manipulation, rather than just genetics, can induce diabetes. As a result, researchers have developed a model for diabetes using environmental and dietary manipulation. This model produces typical results of insulin resistance, hyperinsulinemia, and diabetes. Studies have also shown that streptozotocin (STZ) is capable of inducing diabetes in mice, producing mild to severe types of diabetes depending on the dosage and number of injections administered. Thus, varying doses and numbers of injections have been reported for the development of diabetes mellitus in mice. Streptozotocin is a toxic compound due to its glucosamine nitroso-urea nature, which can damage the DNA of cells. This DNA damage leads to the activation of ADP-ribosylation, which is thought to be an important factor in the induction of diabetes in animals. Diabetes are characterized by abnormalities that result in the malfunctioning of beta cells in the pancreas. In our study, STZ-induced mice showed changes that were indicative of the progression of diabetes. The liver is an essential organ for regulating glucose metabolism.

However, there are differences in food type, duration of diet and STZ dose. As an example, in the

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study of Zhang *et al.*, 2008 the rats were fed with normal chow diet for 4 weeks and then received single injection 45 mg/kg of STZ and Chattopadhyay *et al.*, 1997 used single dose of STZ i.p injection 180mg/kg to produce diabetes on the other side diet usage was also variable. The number of environmental factors has been implicated for pathogenicity of diabetes In the food types; cow's milk has received special attention because it is early int.roduced into the diet or used for replacement for breast milk in rare conditions where breast milk is contraindicated.STZ acts as an toxin for pancreatic beta cell and results in rapid and irreversible necrosis of cells (Arora *et al.*, 2009 (liver)) because multiple low doses of STZ partially destruct the beta cells of pancreas and thereby triggering an inflammatory process that leads to lymphocyte and macrophage infiltration, and whereas a single dose of STZ has been demonstrated to produce complete destruction of beta cells (Kolb, 1993). This study investigated the consequential and persistent effects of A1A1, A2A2 and A1A2 milk consumption on STZ-induced diabetes in experimental mouse model.

MATERIALS AND METHODS

Animals and ethics: For the present study, 28 male mice (C57BL/6) of 6 weeks of age were procured from CSIR-Indian Institute of Integrative Medicine (CSIR-IIIM), Jammu. The mice were transferred to small animal house facility of ICAR-NDRI, Karnal on 15-Jan-2018 for conducting the animal trial. All the animal experiments were conducted after obtaining permission from the Institutional Animal Ethics Committee (IAEC) ICAR-NDRI and all the guidelines of IAEC, under the committee for the purpose of control and supervision of experiments on animals (CPCSEA), were strictly followed.

Animal groups, experimental diet and study period: The animals were given a period of two weeks for acclimatization before the start of experiments. During the acclimatization, animals were fed on normal chow ad libitum and had free access to water. Mice were housed in ventilated plastic cages with soft husk bedding under 12h light/12h dark conditions at controlled room temperature (22 ± 2°C). These conditions were maintained throughout the study period. After acclimatization, the animals were grouped randomly into four groups (control, A1A1, A2A2, and A1A2). Milk based diets were prepared using spray dried milk samples from Karan Fries cows with all the three genotype (A1A1, A1A2 and A2A2). The composition of diets used in the animal trial is summarized in table 1. Control animals were fed standard chow diet while the treatment groups were fed milk based diet. Dietary components like corn starch and sucrose were purchased from a local supplier, unless mentioned otherwise. The animals were given free access to water with their respective diets (ad libitum).

Experimental design: Mice were divided into total 8 groups out which 4 groups were treated with STZ and another 4 groups remained untreated. Six groups (3 untreated normal and 3 STZ-treated mice group) were fed with A1A1, A2A2 and A1A2 genotypes of milk exclusively and rest 2 groups (untreated and STZ treated mice group) fed with chow diet (Table 1). Diabetes was induced by STZ (Streptozotocin) (dissolved in 0.1 M sodium citrate buffer at pH 4.4)at a dose of 45mg/kg of body weight was injected for consecutively 5 days (i.p) intraperitoneally to bring about hyperinsulinemia. After 3 months of trial 3 animals from each group of animals were sacrificed and blood was collected from the eye orbit of mice for the biochemical estimation. After blood collection, sample was allowed to clot and centrifuged at 2000 r.p.m for 15 min. and the serum, obtained was used for analysis. serum was analyzed for biochemical parametres viz. liver functions test (ALT, AST, total protein, albumin), Kidney function test (Urea) and triglycerides were measured using automatic biochemical analyzer (BS 120, DRDO, Chandigarh).

Table 1. Composition of experimental diets used in the animal trial

Ingredients (g/100g)	Control (chow) diet	Milk powder based diet
Milk Powder	—	68.05
Soy Protein	14	—
L-Cystine	0.18	0.18
Corn Starch	49.56	13.91
Maltodextrin	12.5	4.59
Sucrose	10	3.67
Soybean Oil	4.0	—
Cellulose	5.0	5.0
Choline Bitartararte	0.09	0.09
Mineral Mixture (AIN-76) (Teklad, Madison, WI, USA)	3.5	3.5
Vitamin Mixture (Teklad, Madison, WI, USA)	1.0	1.0
t-butyl hydroquinone	0.0008	0.004
Total energy (kcal/g diet)	3.81	4.87

Their serum samples were subjected to biochemical analysis to look for any correlation with respect to A1A1, A2A2, A1A2 milk powder and STZ treated mice.

RESULTS AND DISCUSSION

The data of the current study shows linked relation of different laboratory parameters of liver for different genotypes under varying conditions of control and STZ. The study lists different genotypes that were studied, including A1A1, A1A1 + STZ, A2A2, A2A2 + STZ, A1A2, and A1A2 + STZ. STZ referring to STZ, a drug commonly used to induce diabetes in experimental animals. In the context of diabetic state, it can be observed that the serum alanine aminotransferase (ALT) levels are more impacted than the aspartate aminotransferase (AST) levels, since the controls treated with streptozotocin (STZ) exhibited elevated serum ALT levels in comparison to the controls that were not treated with STZ. Mice fed with A1A1 and A1A2 milk exhibited statistically significantly higher serum AST levels compared to the control group, unlike those fed with A2A2 milk. Despite the fact that the values of serum AST in the A1A1 and A1A2 group were already elevated compared to their control, the values significantly increased in both cohorts of mice induced to develop diabetes using STZ and then fed with A1A1 and A1A2 milk, respectively. Serum AST and ALT values were lower than the control in the blood of mice fed with A2A2 milkbased diet but the laboratory values became significantly higher in the cohort of mice treated with STZ and then fed with the same diet thus diabetic state may exacerbate the impact of the A2A2 milk-based diet on liver enzymes. Almost similar pattern was observed in the serum ALT levels in response to A1A1 and A1A2 milk-based diet. A2A2 milk-based diet had a greater impact on liver enzyme levels in diabetic mice compared to non-diabetic mice. This indicates that diabetic mice possessed a genetic background that facilitated the diet's effects. The exact nature of this genetic background remains unclear and would require further investigation. Nonetheless, the results imply that the interplay between genetic factors and dietary interventions can significantly influence liver function in mice with diabetes. The results of the study indicate that the A1A1 and A1A2 milk-based diets had a more pronounced effect on liver enzyme levels, as evidenced by significantly higher AST and ALT values observed in both diabetic and non-diabetic groups of mice. Yakhchalian *et al.,* 2018;

Table 2. Division of study groups

Non-STZ treated				STZ treated			
Group-1 (control)	Group-2	Group-3	Group-4	Group-5(control)	Group-6	Group-7	Group-8
Chow diet	A1A1 diet	A2A2 diet	A1A2 diet	Chow diet	A1A1 diet	A2A2 diet	A1A2 diet
N=3	N=3	N=3	N=3	N=3	N=3	N=3	N=3

Table 3. Values are expressed as mean ± SE

Parmeters		Control	Control + STZ	A1A1	A1A1 + STZ	A2A2	A2A2 + STZ	A1A2	A1A2 + STZ
TP	Mean	5.367	5.877	5.267	5.367	5.38	5.433	5.433	5.2
	SEM	0.1981	0.0622	0.0333	0.0333	0.1474	0.0881	0.0881	0.1
ALB	Mean	2.667	2.6	3	3.033	3.233	3.2	2.9	3.3
	SEM	0.0666	0.0577	0.1155	0.0333	0.0881	0.1528	0.0577	0.0577
TG	Mean	128.2	254.3	293.8	351.6	212.4	247.8	236.3	318.7
	SEM	1.088	1.167	2.621	2.794	2.966	2.485	1.214	2.963
ALT	Mean	82.9	91.67	93.48	101.7	73.55	93.7	96.25	109.3
	SEM	1.626	0.8819	1.671	1.202	1.103	0.9539	1.315	1.524
AST	Mean	129	133.8	147.9	244.9	131.3	147.7	152.6	253
	SEM	1.528	0.7446	1.726	2.022	1.25	1.369	2.589	1.732
UR	Mean	46.79	46.97	48.88	51	46.97	50.86	63	53.91
	SEM	1.661	1.573	0.7755	0.5774	0.6676	2.381	0.5978	1.491
UA	Mean	3.133	3.2	3.1	3.133	3	3	4.725	4.933
	SEM	0.0881	0.1155	0.0408	0.1333	0.108	0.1528	0.2428	0.0333

Hamadi *et al.*, 2012. The research conducted by Salih *et al.*, 2014 aimed to investigate the impact of STZ-induced Diabetes Mellitus on liver function in mice. The study found that a single injection of STZ caused a significant increase in blood glucose levels, liver weight/body weight ratio, levels of ALP, AST, ALT, bilirubin, and cholesterol. Although the activity of ACP showed no significant change, serum LDH was observed to increase significantly by the second and fourth week but decreased at the sixth week.Based on these findings, the study concluded that STZ-induced Diabetes Mellitus affects the biochemical function of the liver and causes disturbance in liver enzyme levels.AST is found in the liver, heart, skeletal muscle, kidneys, brain, and red blood cells. Liver functionality is measure by calculating the levels of serum AST (Aspartate transaminase), ALT (alanine transaminase), and their ratio (AST/ALT ratio). AST is commonly measured enzyme for liver's physiological assessment as it is more specific to liver. Elevated ALP level depicts the pathology of bone, liver or certain malignancies. Levels of ALP have been measured in multiple studies of induced diabetic mice (Saeed *et al.*, 2008). Azimi *et al.*, 2022 also reported the elevated serum levels of AST. Another study by Zafar *et al.*, 2010 that also revealed significant increase in plasma glucose level of diabetic rats in comparison to control group rats. Similarly transaminase enzymes (AST and ALT) levels were also increased in the study group**.** The same response was seen in study done by Saeed *et al.*, 2008 and Azimi *et al.*, 2022 in separate studies. Thus it guides the clinicians and researchers to find pathology related to metabolic adaptive response. STZ is well known to damage hepatic cellular function of rats that may also be depicted by elevated AST, ALT and ALP levels (Yazdi *et al.*, 2019).

These findings suggest that the A1A1 and A1A2 genotypes may be more sensitive to dietary interventions than the A2A2 genotype. However, additional research is necessary to confirm and further investigate these observations. No significant changes in total proteins were observed in the serum of both diabetic and non-diabetic mice when fed with A1A1, A2A2, and A1A2 milk-based diets. The serum albumin levels in the mice fed with A1A2 milk were higher than the control group, but the values were even higher and statistically significant in the mice that were treated with STZ before the feeding trial. In the trial, it was observed that the controls treated with STZ exhibited higher levels of serum triglycerides, indicating a robust association between

hypertriglyceridemia and diabetic state. These findings suggest that the diabetic state exerts a significant influence on the lipid profile of the subjects under study. The serum triglyceride levels in the blood of experimental mice were significantly elevated in all the cohorts fed with A1A1, A2A2 and A1A2 milk-based diet as compared to the control group. However, the increase was more prominent in the mice that were induced to be diabetic with STZ prior to the feeding trial. This may be attributed to the high fat content of milk-based diet, which tends to predispose to hypertriglyceridemia, a state that is further exacerbated in diabetes.The triglycerides of the body can convert into glucose and can also be stored in adipose tissue (fat cells). Liver is center for gluconeogenesis that is process of converting triglycerides into glucose. Correlation of plasma lipid and glucose are linked to T2DM and insulin resistance that have been explained in multiple literature due to which these were overlooked to find the further metabolic pathways in diabetic state. Abnormal lipid levels in diabetic state have been associated with high levels of plasma triglycerides and LDL with low levels of HDL. Hamadi *et al.*, (2012) have illustrated the insulin deficiency and elevated plasma triglyceride levels in STZ induced mice. The levels of serum urea and uric acid in both diabetic and non-diabetic mice were elevated compared to the control group when fed with A1A2 milk-based diet, implying the presence of unidentified factors in the milk which require further investigation, potentially contributing to renal damage.

In a human study it was found that older adults who consumed more low/reduced fat dairy had lower odds of developing chronic kidney disease (CKD), and increasing dietary calcium intake was also associated with reduced CKD risk. Those in the second quintile of low/reduced fat dairy consumption had 49% reduced risk of CKD 10 years later. (Gopinath *et al.*, 2016). Although some studies on rat models suggest that oxidative stress is a potential link between obesity and renal damage, there is a scarcity of direct studies demonstrating the relationship between milk-based diets, diabetes, and renal damage, with inconclusive results (La Russa *et al.*, 2019).

According to a systematic review of prospective cohort study, although limited, current reports

generally suggest a favorable association between dairy consumption and renal health in the general population, yet more studies are needed to determine whether this relationship is modulated by dairy subtype or fat content (Eslami *et al.*, 2018). In a latest study, it was concluded that consuming a high-fat, high-sugar diet was linked to a 46% increase in the likelihood of developing chronic kidney disease (CKD), while following a lacto-vegetarian diet was associated with a 43% reduced

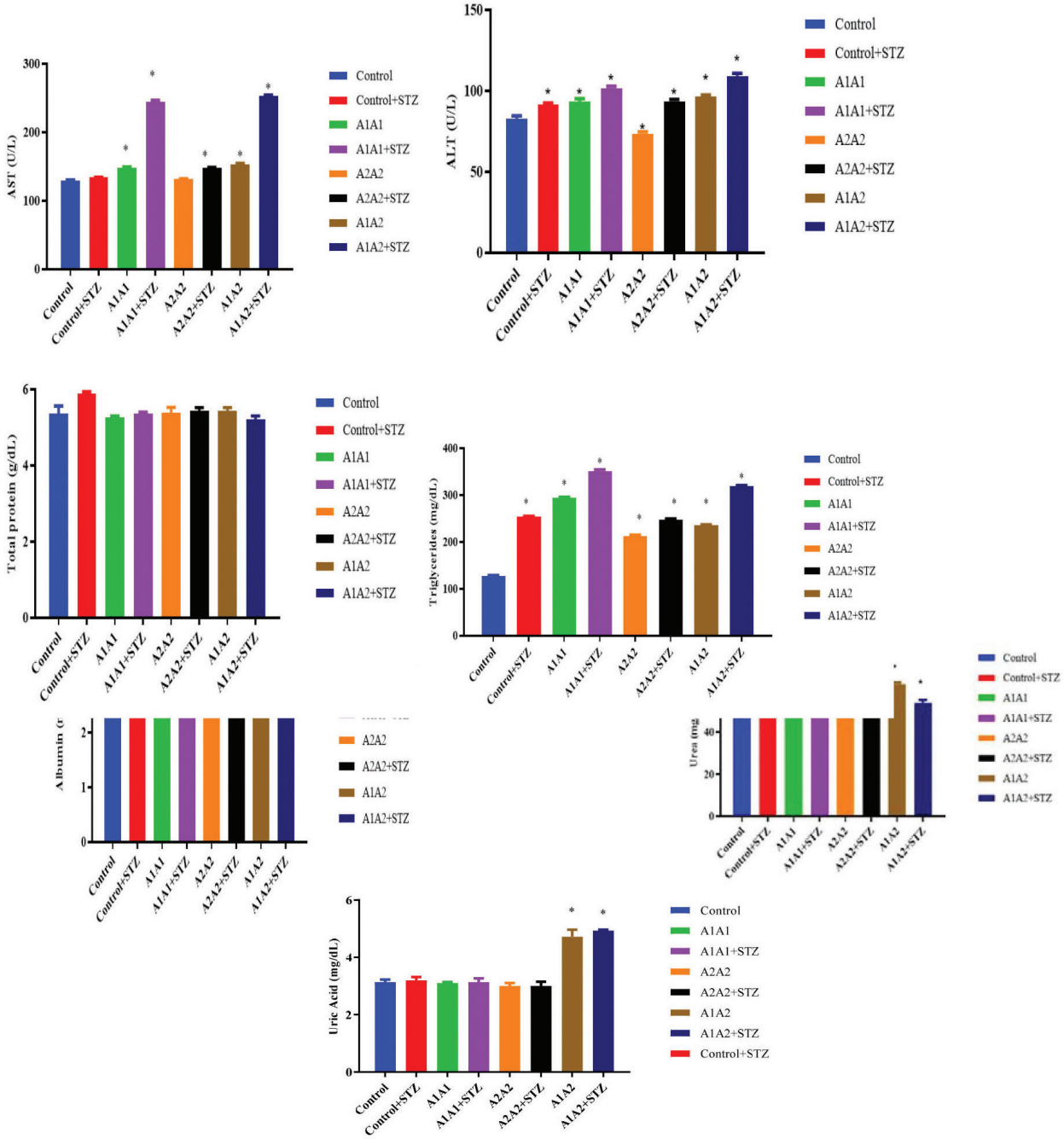

Fig. 1. Values are expressed as mean ± SE. *Significant ($p<0.05$) difference to Control. Level of UA were high in STZ groups compared to all where in A1A1+STZ show high level compared to A2A2 and A1A2, ALT- high in STZ treated groups, AST- high in STZ treated group where low in A2A2 group, Triglyceride(TG) –all groups shows significant changes compared to control, Urea- were high in STZ treated group except A1A1. It might be because of liver disease or damage can lower urea level.

risk of CKD. (Asghari *et al.,* 2018).

The synthesis of beta-casomorphin-7 (BCM-7), a bioactive peptide with potential health implications, is known to be higher in A1 milk when compared to A2 milk, and this disparity is primarily attributed to the variation in the amino acid sequence at position 67 of the beta casein chain. (Singh *et al.,* 2016). Insufficient research on A1 and A2 milk warrants further animal studies to fill gaps and complement human research amidst uncertain evidence. A2 milk, with a protein structure similar to human breast milk and other animal milk, is reported to be easier to digest and absorb than A1 milk, which produces a peptide (BCM-7) causing adverse gastrointestinal effects. (Park *et al.,* 2021; Fernández-Rico *et al.,* 2022; Reddy and Reddy, 2022). No significant changes in total proteins were observed in the serum of both diabetic and non-diabetic mice when fed with A1A1, A2A2, and A1A2 milk-based diets. The serum albumin levels in the mice fed with A1A2 milk were higher than the control group, but the values were even higher and statistically significant in the mice that were treated with STZ before the feeding trial. Blood fats i.e triglycerides are main flexible source of energy. In the trial, it was observed that the controls treated with STZ exhibited higher levels of serum triglycerides, indicating a robust association between hypertriglyceridemia and diabetic state. These findings suggest that the diabetic state exerts a significant influence on the lipid profile of the subjects under study. The serum triglyceride levels in the blood of experimental mice were significantly elevated in all the cohorts fed with A1A1, A2A2 and A1A2 milk based diet as compared to the control group. However, the increase was more prominent in the mice that were induced to be diabetic with STZ prior to the feeding trial. This may be attributed to the high fat content of milk-based diet, which tends to predispose to hypertriglyceridemia, a state that is further exacerbated in diabetes. A study conducted on adult male wistar rats showed that A1 and A2 casein hydrolysate diets did not affect fasting blood glucose or HDL levels, but STZ induced diabetic rats had significantly lower HDL levels compared to healthy rats (Thakur *et al.,* 2020).

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