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Effects of Feeding A1 and A2 Cow Milk-based Diet on Hematological Parameters in Diabetic Mice Model

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ABSTRACT

Background: Milk is essential part of diet across the globe and is a rich source of protein and calcium. Major protein component of milk is casein with beta-casein (β-casein) as the second most prevalent protein in cow milk. β-casein has 15 different genetic variants and of these A1 and A2 have gained research focus. All livestock as well as well human have proline at amino acid position 67 of β-casein, which is referred as A2 variant, but in cattle breeds, other genetic variant called A1 with histidine at amino acid position 67 is also present. This A1 type variant of β-casein or A1 type milk has been implicated as a potential etiological factor in several pathologies.

Methods: The objective of the present study was to evaluate the A1and A2 β-casein variants of cow milk as factors affecting different hematological parameters and other parameters like glucose and insulin in streptozotocin (STZ)-induced diabetic C57/BL6 mice after the induction of diabetes. Diabetes was induced by injecting STZ intraperitoneally at dose of 45mg per kg of body weight for consecutive 5 days. Milk powder prepared from milk with A1A1 and A2A2 genotypes was used for feeding for three months.

Result: After 3 months of feeding trial, it was observed that diabetic mice fed with A1A1 milk (STZ+A1A1) exhibited significantly elevated levels of glucose and insulin. Similarly, the levels of white blood cells, lymphocytes and neutrophils showed significant changes in STZ+A1A1 group compared to control and STZ+A2A2 group indicating the probable association A1A1 milk with inflammatory reaction. However, no significant changes were observed in parameters like red blood cells, hemoglobin, hematocrit or mean cell volume. In the mice group fed with A2A2 milk powder-based diet, no significant change was observed in the observed parameters except lymphocyte percentage which was lower compared to control group. In summary, our results show that A1 form of cow milk might have a proinflammatory effect.

Key words: A1A1, A2A2, Hematological parameters, Milk, STZ (Streptozotocin), β-casein.

INTRODUCTION

Milk having perfect balance of all nutrients required for growth and development is consumed worldwide by infants, children and adults as a high-quality source of protein and calcium. Caseins account for approximately 80% of total milk protein and of caseins β-casein makes up 25-35%. During the course of evolution, different mutations have led to generation of 15 genetic variants of β-casein with A1 and A2 being the most common. The two types differ in their protein structure owing to a substitution of the amino acid histidine to proline, at position 67 (A2: proline; A1: histidine). The specific codon conversion of proline to histidine leads to key conformational changes in the secondary structure of expressed β-casein protein and different bioactive peptides (opioid peptides) released upon its digestion depending upon the genetic variants present (Cieślińska *et al.,* 2012). The opioid peptides generated upon gastrointestinal proteolytic digestion of A1 type milk (histidine at position 67) is beta-casomorphin-7 (BCM-7, while the peptide released from A2 milk (histidine at position 67 in β-casein) is BCM-9. These opioids can cross the epithelial layer and are then free to exert their physiological effect(s) on various tissue types and cells by participating in cellular pathway by virtue of being “atypical” opioid peptides. BCM has morphine like properties that includes a good affinity for an opioid receptor, especially the μ-opioid receptor (MOP), which are widely distributed over cell surfaces of gastrointestinal tract, immune cells, pancreatic cells and various cells in central nervous system and are thus able to exert opiate-like effects by affecting the nerve system and gastrointestinal functions. The BCM-9 is also an opioid agonist but with lesser affinity for MOP. BCM7 has been implicated in many illnesses, including heart disease, type-

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l diabetes mellitus (T1DM) and sudden infant death syndrome. Both *in vitro* and *in vivo* studies have suggested the absorption and transport of BCM-7 across epithelial barrier of small intestine and its implication in different diseases, however, in the case of BCM-9, there are no studies suggesting the trans-epithelial movement. Genotypes with respect to A1/A2 allele of β-casein gene can be either of A2A2, A1A1 or A1A2 type with equal dominance of both the alleles in all the genotypes (Behera *et al.,* 2018).

Frequency of A1/A2 variants differs across the globe (Mukesh *et al.,* 2022) and hence it is important to study the effect of these variants or bioactive peptides released thereof on general human health. It is suggested that BCM-7 released during digestion of milk with A1 β -casein activate mu (μ) receptor that suppress our immune system and produce autoantibody against pancreatic beta cells leading to progression towards diabetes.

Enhanced oxidative stress and changes in antioxidant capacity in both clinical and experimental forms of diabetes are the key mechanisms in the pathogenesis of diabetic complications. Streptozotocin (STZ) induces diabetes by providing relevant ground that induces hyperglycemia caused an endocrinal alterations and oxidative stress. STZ acts as a toxin for pancreatic β-cell and results in rapid and irreversible necrosis of cells (Arora *et al.,* 2009). Multiple low doses of STZ destruct the β-cells of pancreas partially, thereby triggering an inflammatory process that leads to lymphocyte and macrophage infiltration, whereas a single dose of STZ has been demonstrated to produce complete destruction of β-cells (Kolb, 1993). Since diabetes is induced by environmental manipulation rather than only the genes. Using this strategy of environment and dietary manipulation we developed an ideal model for diabetes that shows typical results of insulin resistance, hyperinsulinemia and diabetes. It has been reported that STZ can induce mild to severe types of diabetes depending upon the dosages used and numbers of injections. We tried to find the best protocol and the models most similar to human disease; multiple low doses of STZ diabetes in mice believed to resemble human T1DM (Kolb, 1993). Further, apart from STZ dose and duration, other environmental factors including different food/supplements has been implicated for pathogenicity of diabetes (Jun and Yoon, 2001). Zhang *et al.* (2017) revealed that Hericium erinaceus intracellular polysaccharide purified fractions from mycelia of H. erinaceus SG-02 has antihyperglycemic and protective effects in STZ-induced diabetic mice. Similarly, in a feeding intervention study Chia *et al.* (2018) demonstrated that dietary A1 β-casein may affect glucose homeostasis and T1D progression.

In the food types; cow's milk has received special attention because it is part and parcel of human diet and also used for replacement for breast milk in rare conditions where breast milk is contraindicated. This investigation is often sought-after inducing diabetes and tries to find out how hematological markers would change after STZ induced diabetes in C57/BL6 mice on feeding of A1 or A2 type milk. Whole blood components like white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT) and globulin, are the indices to reflect well-being and good immunity of animals and human. These parameters were measured in both control and diabetic groups and comparisons between two groups was performed to assess the study the effect of A1 or A2 milk.

MATERIALS AND METHODS

Experimental design

For the present study a total of 28 Male C57BL/6 mice of six years of age were acquired from CSIR-Indian Institute of Integrative Medicine (CSIR-IIIM), Jammu.All the mice were shifted to small animal house facility of ICAR-National Dairy Research Institute (NDRI), Karnal on 15-Jan-2018 for conducting the animal trials in accordance with the guidelines of Institutional Animal Ethics Committee (IAEC), ICAR-NDRI. All the guidelines of IAEC, under the committee for the purpose of control and supervision of experiments on animals (CPCSEA), were strictly followed.

In the current study, hyper insulinemic state was induced in C57BL/6 mice by injecting STZ (dissolved in 0.1 M sodium citrate buffer at pH of 4.4) intraperitoneally at a dose of 45 mg per kg of body weight for consecutive 5 days. Enrolled experimental mice were divided into four groups; one group was kept as control and fed with chow diet; other three groups were treated with STZ. Out of three groups treated with STZ, two groups were fed with milk powder diet prepared from milk with A1A1 (A1 milk) and A2A2 genotypes (A2 milk) with respect to β-casein A1/A2 variant, while the fourth group was provided with chow diet but no STZ treatment (Table 1).

The diet was balanced in terms of energy and macronutrient content. Composition of milk powder base and chow diet is described in Table 2.

Hematological parameters

The fasting blood glucose levels were recorded weekly. The tail vein was punctured to collect blood and glucose levels (whole blood) determined using instant glucometer (ACCU-CHECK ® active, Roche Diagnostics, Germany). For other parameters, total 12 mice (03 animals from each group-1, 2, 3 and 4) were sacrificed at 03 months of study and whole blood from each sacrificed mice was collected from the retro-orbital sinus. Collected whole blood was allowed to clot and centrifuged at 2000 rpm for 15 minutes. Obtained segregated serum was analyzed by ELISA kit (cloud-clone corp., USA) in accordance with the manufacturer's instructions, to illustrate the insulin in serum samples of the sacrificed mice. For estimating hematological parameters, automatic hematology analyzer was used and various parameters like red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), mean cell volume (MCV), white blood cell (WBC) count and WBC differential (lymphocytes and neutrophils) were analyzed.

RESULTS AND DISCUSSION

Effects of STZ on blood glucose and serum insulin

Foods and nutrients play vital roles in normal biochemical functioning of the body and immune system. Studies on A1 and A2 milk have increased since the year 2010 (Jiménez-Montenegro *et al*., 2022) especially in the field of food science technology and agriculture depicting the adverse health effects with A1 β-casein milk. Milk with A1 variant of β-casein has been associated with different diseases like Schizophrenia, Diabetes, Acquired heart diseases and Autism *etc*. To test whether A1/A2 β-casein variants would affect development of diabetes in multiple low-dose streptozotocin (MLDSTZ) model of disease, mice were fed with milk-based powder diet prepared with different genotypes of β-casein that is A1A1 and A2A2 over a period of three months. Mice from both the diet groups A1A1/A2A2 and control group received STZ injections intraperitoneally for five days to induce diabetes. Induction of diabetes through the administration of a β-cytotoxic drugs like STZ is well pronounced in the literature (Unwin *et al.,* 2002) and is the first choice for induction of diabetes in experimental animal models (Lenzen *et al.,* 1996; Arison *et al.,* 1967). The mechanism involved is specific necrosis of the pancreatic β-cells study by Cetkovic-Cvrlje *et al.* (2017), wherein C57BL/6 mice treated with low-and high-dose of STZ, showed clear signs of diabetes. The suggestive mechanism was destruction of β-cells by STZ.

Table 1: Grouping of mice based on STZ treatment and diet.

Control (Non-STZ treated)	STZ treated		
Group-1 (Control)	Group-2 (STZ-Control)	Group-3	Group-4
Chow diet N=07	Chow diet N=07	A1A1 diet N=07	A2A2 diet N=07

Table 2: Composition of diet given to experimental mice.

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

High blood glucose levels are the main characteristic of Diabetes mellitus. In our study it was observed that mean whole blood glucose level was high in all the STZ treated mice groups compared to the controls. Average glucose level was 206.7±1.8 and 167 ±1.4 in STZ-control and control group respectively. In the same groups of mice, mean serum insulin levels were 4.42±0.57 IU/L (STZ-control), 1.827±0.27 IU/L (control) whereas highest insulin level (5.548±0.53 IU/L) was observed in A1A1+STZ group, shown in Table 3 and Fig 1. The differences in values were high and statistically significant suggesting that A1A1 milk might have some component that triggers a mechanism leading to hyperinsulinemia. The result of the present study indicated that STZ treatment and STZ+ A1A1 milk-based diet significantly elevates the blood glucose level and leads to diabetes. In a study by Yadav *et al*. (2020) involving feeding of A1/A2 milk in male BALB/c revealed augmented airway hyperresponsiveness with increasing concentration of bronchoconstrictor, elevated levels of IL-4, IL-5 in bronchoalveolar lavage and serum, increased IgE, IgG levels along with increased infiltration of lymphocytes and eosinophils in mice fed with A1 milk. It was concluded that A1 variant of cow milk spectate proinflammatory effect similar to allergic asthma phenotype on the lungs. A1 milk depicts clinical health hazards as compared to A2 milk in most of the observational, epidemiological and clinical studies on experimental animals and human (Reddy and Reddy, 2022).

According to a study conducted by Jiang *et al.* (2007) most of the diabetic transgenic mice restored normal blood glucose, serum insulin levels and islet cell mass after MLDSTZ. In the current study, glucose level showed

Table 3: Hematological parameters, glucose and insulin levels across different experimental groups.

	Control	Control+STZ	A1A1+STZ	A2A2+STZ
RBC, ×106/µL	7.075±0.2	6.133±0.17	5.933±0.2	7.467±0.2
HB, g/dL	10.58±04	10.2±1.106	10.03±0.6	10.7±0.15
HCT %	33.68±0.9	36.2±0.50	35.8±1.00	33.27±0.8
MCV, (fL)	48±0.9	45.67±1.7	45.67±1.6	43.33±1.6
WBC, thou/µL	4.8±0.2	12.03±0.1	13.43±1.6	6.033±0.2
Lymphocytes, %	82.75±1.3	83.33±0.8	83.5±1.25	72.67±13
Neutrophils, %	7.25±0.85	14±0.57	10.33±03	9±0.57
Glucose, mg/dL	167.9±1.4	206.7±1.8	306.1±1.17	260.9±1.8
Insulin, IU/L	1.827±0.27	4.425+0.57	5.548±0.53	2.587±0.22

statistically significant ($p \leq 0.05$) higher values which is supported by a study in which glucose levels were decreased in biotin treated diabetic mice as compared to untreated group (Aldahmash *et al.,* 2016) that happened because deficiency of biotin decreases pancreatic glucokinase activity and insulin secretion from pancreas. STZ causes destruction of β-cells and thus have potential to cause diabetes which is also revealed in another study done by Cetkovic-Cvrlje *et al.* (2017) in which, a strong diabetogenic potential of BPA was found, as an aggravation of T1DM development in low-and high-dose of STZ-treated C57BL/6 mice that were sub-chronically exposed with BPA. In another study done by Kim *et al.* (2018), Chrysanthemum zawadskii extract supplementation in STZ-induced type-1 diabetic rats and STZ + high fat diet-induced type 2 diabetic mice enriched insulin resistance and glycemic control with decreased hemoglobin A1c (HbA1c) levels in serum. In another study, C57BL/6 mice were induced to diabetic state by STZ and plasma insulin levels were significantly low (Goodarzi *et al.* 2019).

Effect of STZ on Hematological parameters

Whole blood components like WBCs, RBCs, Hb and indices like HCT and Packed Cell Volume (PCV) are the indicators to reflect the well-being and good immunity of animals and human. These hematological indices are also the good indicators to reveal the environmental effect on animals and to comprehend their physiological status with respect to nutrients and quality of feed ingested. In the present study, changes in the level of some of the hematological parameters were observed in the diabetic mice. The level of Hb, an iron-containing conjugated protein that performs the physiological function of transporting oxygen and carbon dioxide, did not show any significant changes between STZ treated diabetic and control groups which is in consistence with the study done by Jothi *et al.* (2016) and El-Sayed *et al.* (2019) where experimental animals did not suffer from depressed respiratory capability indicating that the oxygen-carrying capacity of the blood was not affected. However,

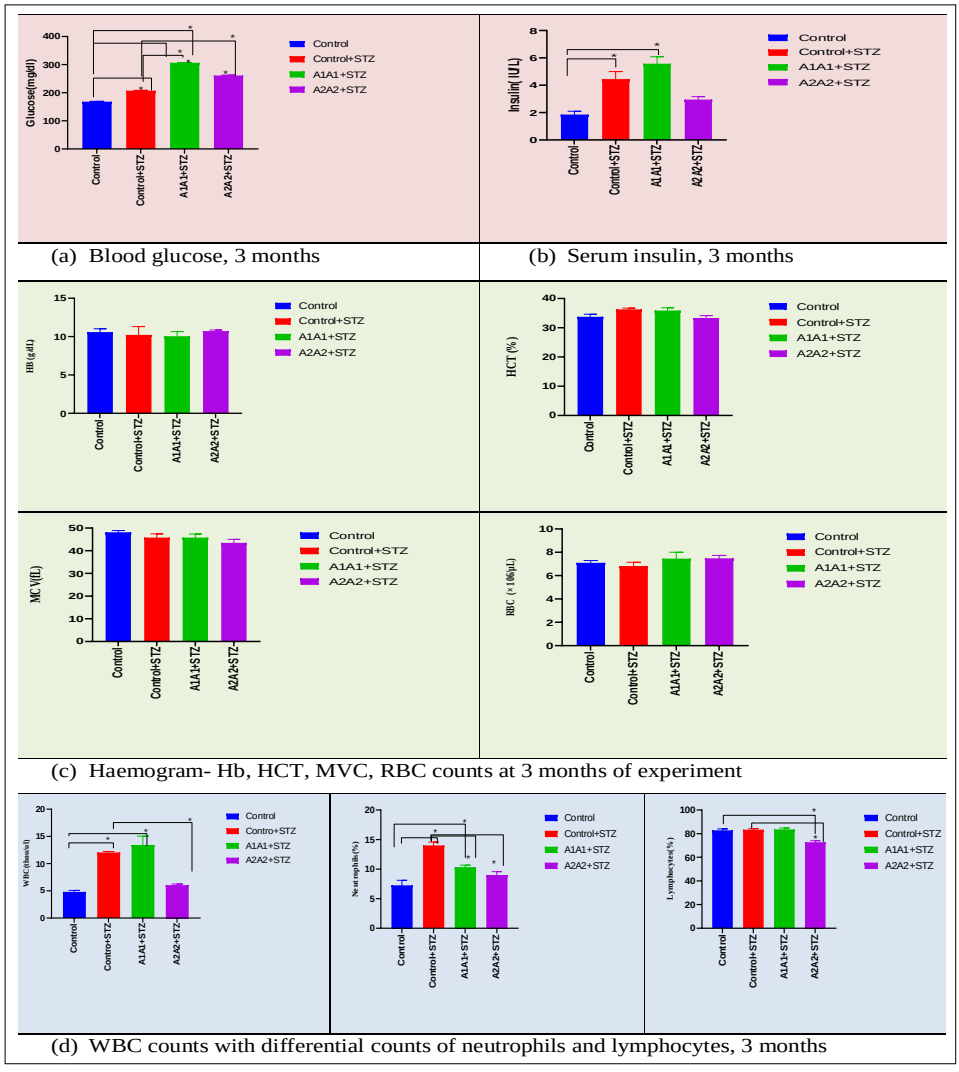

Fig 1: Levels of glucose, insulin and other Hematological parameters and their level of significance across different experimental groups.

Akpan and Ekaidem (2015) observed immunological and hematological alterations in STZ treated (intraperitoneal injection of 60 mg/kg) rat mainly due to oxidative stress. Significant reduction was observed in RBC, Hb, PCV along with erythrocyte function indices like Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH) and Mean corpuscular hemogloblin concentration (MCHC) while level of WBC, neutrophil, lymphocyte, platelet increased in diabetic control compared to the normal control. In the same study, effect of feeding of leaves of Vernonia amygdalina and Gongronema latifolium on modulation of hematological parameters was also investigated. The analysis clearly indicated the modulation of hematological parameters by specific diet, with reduced WBC and platelet but significantly increased ($p<0.05$) RBC, Hb, PCV, MCV, MCH and MCHC in the diabetic treated group compared to diabetic control.

Keskin *et al.* (2016) also demonstrated the decreased levels of RBC counts, PLT, Hb and HCT without change in parameters like MCV, MCH, MCHC in STZ induced diabetic rats. In the current study, significant changes were not observed in RBC indices (Hb, HCT and MCV) across all four groups (Control, STZ-Control, A1A1+STZ and A2A2+STZ). Total average RBC counts in the control and STZ-Control of current study were 7.1±0.2 mil/µL and 6.133±0.17 mil/µL respectively and no significant changes were observed with respect to experimental group that is A1A1+STZ and A2A2+STZ. Average Hb, HCT, MCV values in the 'control' and 'STZ-Control' was 10.58±0.4 g/dL and 10.2±1.106 g/dL; 33.68±0.9 and 36.2±0.50% and 48±0.9 fL and 45.67±1.7 fL respectively. Though some change in the values was observed across groups, these were not significant.

Overall, in our study, no effect of A1/A2 based diet was observed on C57BL/648 mice across different groups which is in line with study of Thakur *et al.* (2020), wherein no effect of feeding A1 and A2 cow milk derived casein hydrolysates was observed on the blood biochemical profile in diabetic model of rats. Similarly, in the study, where gilt pigs were fed with A1A1 or A2A2 milk over a period of six weeks, no change was observed in blood indices viz. WBCs, RBCs, Hb, platelets (Kaminski *et al.,* 2012).

In the current study, significant changes were observed in the level of WBC and lymphocytes across the different experimental groups. WBC level was higher in STZ-Control (12.03±0.1 thou/µL) and A1A1+STZ (13.4±1.6 thou/µL) while lower levels were observed in A2A2+STZ (6.033±0.2 thou/µL) and control (4.08±0.2). Increase in WBC levels might be attributed to oxidative stress as suggested by Shurtz-Swirski *et al.* (2004). In another study, raised WBC levels were observed in diabetes due to oxidative stress along with induction of inflammatory process leading to infiltration of lymphocytes followed by the onset of insulin deficiency (Kolb, 1993). Regarding lymphocyte per cent, least values of 72.67±1.3% was observed in A2A2+STZ which was significantly different from control+ STZ (83.33±0.8%) and A1A1+STZ (83.5±1.25%) and control (82.75±1.3). Similarly, neutrophil percentage showed significant changes in A2A2+STZ (72.67±1.3%) compared to A1A1+STZ (83.5±1.25%) and Control+STZ (83.33±08%). Similar to our study, Essiet *et al.* (2020) also found high WBC, neutrophils counts and low lymphocytes in diabetic induced mice compared to their controls. In diabetes mellitus, increased production of free radicals especially reactive oxygen species (ROS) occurs and results into oxidative stress. Destruction mediated by such free radicals results in protein denaturation and breakdown of DNA strands. Therefore, maintaining redox balance is important in the context of disease prevention. Inflammation plays an important role in pathophysiological changes in diabetes mellitus. Some inflammatory markers are used to predict the risk in developing diabetes mellitus and these cytokines respond by our immune system. However further studies are required to know the association of A1A1 milk and induction of inflammatory reactions.

CONCLUSION

STZ induced mice showed some changes in the WBC, lymphocyte and neutrophil count. Feeding of A1A1 milk-based diet further aggravated these changes. However, all other hematological parameters were unaltered in mice fed with A2A2 milk-based diet. Considering results of the present study, we can say that A2A2 diet given to STZ treated mice group does not cause any abnormalities while A1A1 diet in the STZ diabetic mice causes few abnormalities related to the inflammation.

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Ethical approval

Animal trials was conducted accordance to the guidelines of Institutional Animal Ethics Committee (IAEC), ICAR-NDRI.

Declaration

The authors declare no competing interests.

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