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Evaluation of Feeding Effects of A1 and A2 Cow Milk-Based Diet on Hematological Parameters in Obese Mice Model

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ABSTRACT

Beta-casein is one of the four caseins that make up approximately 80% of cow's milk proteins. Of the 15 known genetic variants of β-casein, A1 and A2 are the most prevalent. These two variants differ by a single amino acid at position 67, where the A2 variant contains proline, while in the A1 variant, proline is replaced by histidine. Based on A1 and A2 β-casein variants cow milk is categorized as A1 and A2 milk receptively. Structural variations due to change of amino acid result in generation of different bioactive peptides during gastrointestinal protease digestion and beta casomorphin 7 (BCM-7) released from digestion of A1 milk has been implicated as risk factor for human health. In the present study, we analyzed the effect of A1/A2 milk powder-based diet on hematological parameters in obese model of C57BL/6J mice (fed with high-fat diet). Mice in different diet groups were fed with milk powder-based diet prepared from milk with three different genotypes (A1A1, A1A2 and A2A2) with reference to A1/A2 allele of beta casein for 90 days. The observations revealed significant changes in blood glucose, Hb, HCT, WBC and RBC count in mice from control vis a vis high fat diet group. HFD+A1A1/A1A2 group also showed significant alterations in blood glucose changes compared to control. Across the milk powder-based diet groups, blood glucose level and WBC count in HFD+A1A1 group was significantly higher than HFD+A2A2 group pointing towards the potential pro-inflammatory response. No changes were observed in Hb, hematocrit, MCV or RBC count across milk powder-based diet groups. However, this is a preliminary study that would need further exploration.

Key words: High-Fat Diet, Hematology, A1/A2 milk, Genotypes, Mice, Glucose

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INTRODUCTION

Milk is included in the diets of infants, children and adults worldwide, as it has the most abundant protein and trace element known to be essential in human nutrition (Laakkonen and Pukkala, 2008). It not only provides good energy and protein for most people, but also contributes to the body's needs for calcium, magnesium, selenium, B-complex vitamins (thiamine, riboflavin, niacin, vitamin B6 and folate), vitamin A, vitamin C, magnesium and zinc (Jelen, 2009). Dairy cows provide the largest amount of milk, at 600 million tons (83% of all milk). Milk contains lipids, proteins, amino acids, vitamins, minerals, immunoglobulins, hormones, growth factors, cytokines, nucleotides, peptides, polyamines, enzymes and other biologically active peptides with physiological functions. Bioactive molecules in milk can have a direct and significant effect on human health (Marshall, 2004), therefore milk and dairy products are considered functional food. Among the different components of milk, proteins have been recognized as rich source of biomolecules/bioactive peptides exerting different bioactivities. Milk usually contains about 3.5% protein, of which more than 95% consists mainly of casein (80%) and whey protein (20% more); of all caseins, alpha S1 makes the highest contribution (39-46% of total casein), followed by alpha S2 (8-11%), beta (25-35%) and kappa (8-15%) caseins (Eigel et al.,1984). Whey protein contains two main proteins: alpha-lactalbumin and beta-lactoglobulin. Caseins or casein fractions do not have any identified physiological role, but it's the peptides derived from casein by enzymatic proteolysis that possess various bioactive properties (Jelen and Lutz, 1998). Apart from naturally occurring peptides, diverse array of bioactive peptides affecting milk nutritional properties can originate due to substitutions or deletions resulting from genetic polymorphism. Due to genetic changes, the composition of amino acids changes, resulting in the production of different biomolecules. An important variant found in the β-casein gene is the A1 and A2 variants, resulting from a change in exon VII of the bovine β-casein gene on chromosome 6. It occurs in the substitution of proline (A2 allele, codon; CCT) by the amino acid histidine (A1 allele, codon; CAT) at position 67 (Groves, 1969). There are other variations in beta casein gene but these are either A1 "like" having a common amino acid histidine (His) at position 67 or A2 "like" milk having a amino acid proline (Pro) at position 67, but variations at other positions (Roginski, 2003). This polymorphism (proline to histidine) leads to key conformational changes in the secondary structure of expressed β-casein protein. Due to presence of histidine at amino acid 67 position, gastrointestinal proteolytic digestion (leucine aminopeptidase, elastase and carboxypeptidase Y) of A1 β-casein (raw/processed milk) releases a 7 amino acid bioactive peptide 'opioid' called beta-casomorphin 7 (BCM-7) in small intestine, while in case of A2 milk, proline inhibits the split at this particular location and nine amino acid peptide BCM-9 (Roginski, 2003; Kostyra et al., 2004) is released. The beta-casomorphine-7 has been identified as the "atypical" opioid peptide with properties similar to morphine, and exerts its influence on nervous, digestive, and immune functions via MOR (**μ-opioid receptor)** and DPPIV (Dipeptidyl peptidase IV), genes expression. These **μ**-opioid receptors are present in the neurological system, pancreatic beta cells, endocrine system, and immune system. Binding of MORs with exogenous ligands such as BCM-7 initiates various intracellular pathways that may lead to different health disorder. Due to the generation of BCM-7 peptide, A1 variant of beta casein or A1 milk has been implicated as a potential etiological factor in a number of human diseases including ischemic heart disease, diabetes mellitus-1 and autism, but, A2 variant has not associated with any health disorders (Kaminski et al., 2007; Tailford et al., 2003;Jianquin et al., 2016).

With changing diet style, obesity has become a common health problem these days (Fruh, 2017). Obesity is associated with a general dysregulation of metabolic homeostasis resulting in different health issues (Muoio and Newgard, 2006)and thus is a major field of interest for basic science and clinical research (Tchernof et al., 2013). In the prevailing scenario of obesity, if the ill effects of A1type stand true, consumption of A1 type milk can result in health complicacies in obese persons. In the present study, efforts were made to study the effect of consumption of A1A1, A1A2 and A2A2 milk powder-based diet on hematological parameters in high-fat diet-induced obese C57BL/6J mice. The feeding trail was carried out for 90 days and different hematological parameters including hemoglobin, hematocrit, mean corpuscular volume, red blood cells, white blood cells and glucose levels were compared and evaluated across different groups. Mice model was used for studying the aspects of milk powder-based diet as well as otherwise complications rising due to specific diets for example A1/A2 milk or obesity require decades to develop (Wang et al., 2012).

MATERIALS AND METHOD

Experimental design

A total of 25, 4-week-old male C57BL/6 mice were obtained from CSIR-Indian Institute of Integrated Medical Sciences (CSIR-IIIM), Jammu. All mice were transferred to ICAR-National Dairy Research Institute (NDRI), small animal facility, Karnal and animal experiments were per-

formed in accordance with the guidelines of Institutional Committee on Animal Ethics (IAEC), ICAR-NDRI. Mice were housed (22 ± 2 °C) under 12 h light/12 h dark cycle in plastic cages with husk bedding. These conditions were maintained throughout the study period. After acclimatization, mice were divided randomly into five groups and fed with respective diet for 3 months ; I) Control group: mice were fed with normal chow diet; II) HFD group: mice fed with high fat diet; III) HFD+A1A1: mice were given high fat diet plus milk powder diet prepared from animals with A1A1genotype; IV) HFD+A2A2: high fat diet plus milk powder prepared from animals with A2A2 genotype; V) HFD+A1A2: high fat diet plus milk powder prepared from animals with A1A2 genotype. The A1A1 genotype refers to the homozygous status of A1 allele, A2A2 genotype refers to the heterozygous status of A2 allele while A1A1 genotype refers to the homozygous status of A2/A1 allele of beta casein. Milk powder was prepared by freeze drying the milk collected from Karan Fries cows (available at ICAR-National dairy Research Institute, Karnal) with specific genotypic status. The energy and macronutrient makeup of the diets were standardized.

Mice from Chow, HFD and HFD plus milk powder-based diet groups had their respective diet and water offered *ad libitum*. Chow diet having soy protein was purchased from commercial vendors while HFD (containing 13.2% cow milk ghee) and HFD plus milk powder-based diets were prepared in the lab. Due care was taken to include all the minerals and vitamins along with energy requirements while high-fat diets and high fat plus milk powder diet was prepared in the lab. Mice in all groups remained healthy throughout the course of the study. Overall, animals exhibited normal behavior, and no signs of illness or lethargy were noted.

Hematological parameters

Fasting blood glucose levels were recorded monthly and blood samples were taken from a tail vein. Blood glucose results (whole blood) were recorded using a blood glucose meter (ACCU-CHECK ® **active, Roche Diagnostics, Germany). No adverse effects on behavior were observed in any of the mice after blood collection.** Hematological parameters such as hemoglobin (Hb), hematocrit (HCT), mean cell volume (MCV), white blood cell (WBC) and red blood cell (RBC) counts were measured using an automated hematology machine.

Statistical analysis

Data was analyzed using one-way ANOVA with GraphPad 8.4.3 (GraphPad Software, San Diego, CA, USA) and Tukey's post hoc test. A significance level of p=0.05 was used unless otherwise stated in the figure. Results are expressed as means ± SEM.

RESULTS & DISCUSSION

Milk provides good protein and micronutrients and has many health benefits (Bell et al., 2006). However, a link between disease risk and specific reductions in the beta-casein fraction with the A1 or A2 genes has been identified. There is evidence linking A1 β-casein to different diseases, including type 1 diabetes, CVD, atherosclerosis, autism and sudden infant death syndrome (Laugesen and Elliott, 2003; Virtanen et al., 2000; Tailford et al., 2003; Woodford, 2006; Sun et al., 2003). In the present study, we evaluated the effect of feeding milk powder-based diet prepared from different cows with specific genotypes with reference to A1/A2 allele of beta casein along with high-fat diet for up to 90 days on the male C57BL/6 mice hematological parameters. C57BL/6J mouse model is considered as good model as it mimics well the human metabolic disorders observed in obesity or other related disorders on feeding with specific diets. Buettner et al., (2007) also reported that among different rodent models, diet-induced obesity models represent the best fit for comparison to human obesity related complicacies.

Effect of milk powder-based and high fat diet on the hematological parameters

Alterations in the basic hematological parameters were studied on consumption of high fat diet and diet prepared from milk with A2A2, A1A2 and A1A1 genotype. The hematological parameters were selected as these are the basic indicator of overall health and immunological condition in both people and animals. These indices are also valuable in understanding their physiological condition in relation to type of feed intake. Studies have revealed the effect of diet on the hematological parameters in diet-based mice models. Changes in WBC, lymphocyte and neutrophil count on feeding of milk powder diet prepared from cows with A1A1 genotype with reference of A1 allele of beta casein in Streptozotocin-Induced Diabetes Mice Models has also been reported (Chaudhary et al. 2023). In the present study, effect of high fat diet as such and in combination with milk powder-based diet prepared from milk with different genotypes of beta casein on hematological parameters was evaluated in obese mice model. Obesity has been described as a chronic disease (Donath and Shoelson, 2011) that is often associated with hematological changes (Farhangi et al., 2013).

Different diets and the blood glucose level

Evaluation of mean blood glucose level (mg/dl) in mice from all the five groups that is I) Control; II) HFD group; III) HFD+A2A2; IV) HFD+A1A2 and V) HFD+ A1A1 group, indicated the highest value in HFD+A1A1 (185.5±2.64) followed by HFD+A1A2 (181.2±3.43) groups (Figure 1a). The mean blood glucose level in mice group fed with high fat diet (179.0±4.45) was significantly higher than the mice from the control (157.6±1.81) group. This aligns with the previous reports which indicate the higher blood glucose levels in mice on feeding the high-fat diets due to insulin resistance and metabolic abnormalities. In a study, mice were given a diet rich in ghee, and biochemical analysis showed that blood sugar was 30% higher in the high-fat diet group compared to control group mice (Basheer et al.2023). A study by Winzell et al (2004) showed that rats fed high-fat diet developed glucose intolerance, leading to elevated blood sugar levels. Across the mice groups fed with HFD+A1A1 and HFD+A1A2 milk-based diet, the mean blood glucose level was 185.5±2.64and 181.2±3.43, respectively and all were significantly higher than the mean values for mice from the control group.

The mean blood glucose level of mice fromHFD+A2A2 diet group was significantly lower (171.4±3.84) than that of mice from HFD+A1A1 group but the difference with HFD+A1A2 mice were non-significant (Figure 1a). The comparative evaluation of blood glucose level across the mice groups fed with HFD and milk powder (with specific genotype) based diet indicate towards negative impact of milk powder diet prepared from cows with A1A1genotypeon glucose metabolism. Reports from other researchers have also suggested the association of intake of A1 type beta-casein with impairment glucose metabolism, hence increas-

Table1. Composition of Experimental Diets

S.No.	Ingredients	Control Diet (AIN-93 M) (g/100g)	Milk Based Diet (g/100g) (A1A1/A2A2/A1A2)
1	Protein (Milk)	-	14
2	Soy Protein	14	-
3	L-cystine	0.18	0.18
4	Corn Starch	49.56	13.91
5	Maltodextrin	12.5	4.59
6	Sucrose	10	3.67
7	Lactose (Milk)	-	22.09
8	Cellulose	5	5
9	Soyabean Oil	4	-
10	Fat (Milk)	-	20.88
11	Choline Bitartrate	0.09	0.09
12	t butyl hydroquinone	0.0008	0.002
13	Mineral Mix (AIN-76)	3.5	3.5
14	Vitamin Mix	1	1
		3.81 kcal/g	**4.21 kcal/g**

Table.2.Hematological parameters and glucose levels across different diets.

Diet groups	Glucose (mg/dl)	Hb (g/dl)	HCT (%)	RBC (mil/ul)	MCV (fl)	WBC (thou/ul)
Control	157.6±1.81	9.07±0.43	29.78±0.73	5.42±0.19	51.75±1.00	4.55±0.67
HFD	179.0±4.45	11.50±0.55	37.80±0.25	7.78±0.45	52.00±0.57	8.10±0.49
A1A1+HFD	185.5±2.64	9.99±0.21	31.73±2.68	6.92±0.02	52.25±2.01	8.40±0.25
A2A2+HFD	171.4±3.84	9.96±0.23	30.37±1.81	6.01±0.48	50.33±0.88	5.76±0.51
A1A2+HFD	181.2±3.43	9.97±0.12	33.40±1.61	6.73±0.33	51.00±2.04	7.90±0.40

Control: chow diet; HFD: high fat diet; A1A1: milk powder prepared from milk with A1A1 genotype; A2A2: milk powder prepared from milk with A2A2 genotype; and A1A2: milk powder prepared from milk with A1A2 genotype. Results are shown in Mean + S.E.M.

ing the risk of diabetes (Ho et al., 2014). Further, addition of beta-casein with A1 allele (A1A1 milk) to high-fat diet, may raise blood glucose levels by aggravating the effects of a high-fat diet on blood glucose levels in comparison to high-fat diet alone (Jianqin et al.,2016). On the other hand, A2 allele of beta-casein (milk with A2A2 genotype) does not impair glucose metabolism as the reported blood glucose level (171.4±3.84) was even lower than the HFD group (179.0±4.45). Previous study by our group (Chaudhary et al., 2023) also indicated the significantly higher glucose level in diabetic mice fed with A1A1 milk powder-based diet compared to normal control and HFD control.

The effect of A1 and A2 β -casein in milk on the progression of type 1 diabetes or glucose control is unclear (Thakur et al., 2020), but data suggest that β -casein can stimulate T-cell immunity and anti-inflammatory drugs that cause type 1 diabetes (Monetini et al., 2003). Few reports suggest that A2A2 milk may have positive role in maintaining blood glucose levels, particularly in those with type 2 diabetes (Monetini et al., 2002). The adverse effects of A1 milk have been well reported in different studies. Barnett et al (2014) reported that β-casein depletion has a direct effect on intestinal casein A2 activity via opioid-dependent and non-opioid-dependent pathways.

Hematological analysis for RBC and related parameters

Comparative values of RBC-related parameters, such as the concentration of hemoglobin (Hb), hematocrit (HCT) percentage, mean corpuscular volume (MCV), and red blood cell count are presented in figure 1(b-e) and Table 2. High fat diet also is associated with changes in level of hemoglobin, an iron-containing conjugated protein in red blood cells, responsible for the physiological transport of oxygen and carbon dioxide though the underlying mechanism is unclear. Evaluation of mean hemoglobin count (mg/dl) in mice from all the five groups that is I) Control; II) HFD group: III) HFD+A2A2; IV) HFD+A1A2 and V) HFD+ A1A1 group (Figure 1b), indicated the highest value in HFD group (11.25±0.25) and least value was observed in HFD+A2A2 group (9.96±0.23).The mean Hb level in mice group fed with high fat diet (11.25±0.25) was significantly higher than the mice from the control(9.07±0.43) group. However, the changes across high-fat diets along with A1A1, A2A2, and A1A2 milk powder-based were not significant. Hematocrit, a simple parameter to monitor the response to specific diet or treatment was also evaluated across the different diet groups. It measures the volume of packed red blood cells (RBC) relative total volume of blood. The HCT percentage in the high-fat diet group (37.80±0.25) was considerably higher (Figure 1c).While, there was no discernible change in the hematocrit percentage between the groups of mice fed with high-fat diets along with A1A1, A2A2, and A1A2 milk powder-based diet (31.73±2.68, 30.37±1.81 and 3.4±1.61 respectively) and the control group (29.78±0.73) (Figure 1c). There are different reports indicating change of Hb and HCT in response to high fat diet intake. In a study involving feeding of low and high fat diets to rat, lower hemoglobin concentration and hematocrit was observed in rats fed with high fat diet (21%) than those fed on diets with intermediate (11.9%) and low (5%) fat content (Edozien and Switzer,1977). However, in another study where male C57BL/6mice were fed on high fat diet (60% fat) for 6 months, higher hemoglobin concentration and hematocrit (HCT) percentage was observed in high fat diet group compared to control-fed mice (Maysami et al., 2015). Even in human subjects, increased values for mean Hb count and Hct have been reported among groups with higher body mass index (Jeong et al., 2021)

The mean corpuscular volume (MCV) is a critical measurement for identifying the underlying cause of certain diseases. It gives the value that measures the average size and volume of red blood cells (Brittany et al., 2024). Evaluation of mean MCV level (ft) in mice from all the five groups that is indicated no discernible variation in the Mean Corpuscular Volume (MCV) between the mice fed with high-fat diets along with types of milk powder-based diet withA1A1, A2A2, and A1A2 genotypes (52.25±2.01, 50.33±0.88 and 51.00±2.04, respectively) and either the high-fat diet group (52.00±0.57) or the control diet group (51.75±1.00) (Figure 1d). The results are in line with observations for Swiss mice fed on high fat diet but for 24 weeks which showed no significant change in MCV level, however changes across control and high fat group for hemoglobin and hematocrit were significant (Gotardo et al., 2013). Diet prepared from milk with different genotypes also had no significant effect on MCV values. It indicates that the high fat diet and milk powder-based diets are not associated with character of obesity as a low-grade inflammation that tends to come up with iron-deficiency anaemia (Auskand Ioannou, 2008). Conversely, Maysami et al., (2015) have observed significant increase in mean corpuscular volume level in high-fat-fed mice compared to control group.

Red blood cell (RBC) levels in group following a high-fat diet (7.78±0.45) were considerably higher than in the group following a control diet (5.42±0.19) (Figure 1e). While, there was no discernible change in the RBC levels between the groups of mice fed with high-fat diets along with A1A1, A2A2, and A1A2 milk powder-based diet (6.92±0.02, 6.01±0.48 and 6.73±0.33 respectively) and the control group (5.42±0.19) (Figure 1e).

Hematological analysis for WBC

Comparative evaluation across dietarygroups revealed a significant increase in white blood cell (WBC) counts in mice from obese control (HFD diet group) compared to the normal control (chow diet group) with values of 4.55±0.67and 8.10±0.49 respectively. Across the milk-based diet groups, the WBC count in HFD+A1A1 group was significantly higher than control group and values are higher (8.40±0.25) than HFD group. Conversely, in HFD+A2A2 group WBC count was significantly lower (5.76±0.51) than HFD+A1A1 group (Figure 1f). The observations are consistent with those of Chaudhary et al., (2023), where STZ-treated mice fed an A1A1 milk-based diet exhibited a significantly higher WBC count compared to those on a chow diet. In contrast, mice fed an A2A2 milk-based diet showed no discernible differences in WBC count compared to the control group. High-fat diets are commonly associated with the induction of oxidative stress, which may trigger low-grade inflammation and consequently lead to an increased white blood cell (WBC) count. The present observations are consistent with Maysami et al.,(2015)who have demonstrated obesity induced inflammation resulting in increased WBC counts. Signs of inflammation as indicated by increased WBC

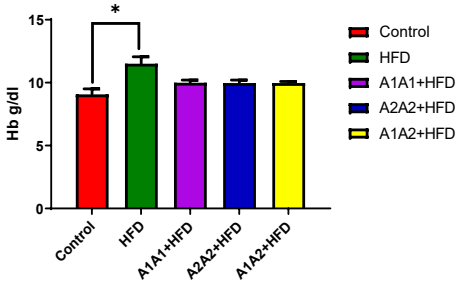

Figure1 (a)

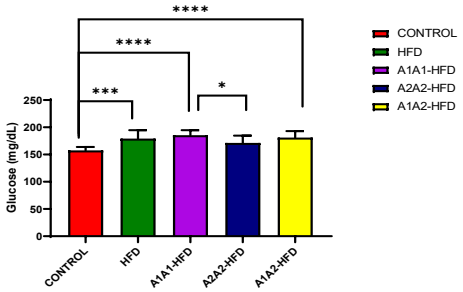

Figure1 (b)

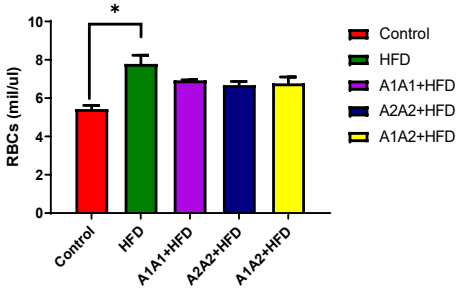

Figure1 (c)

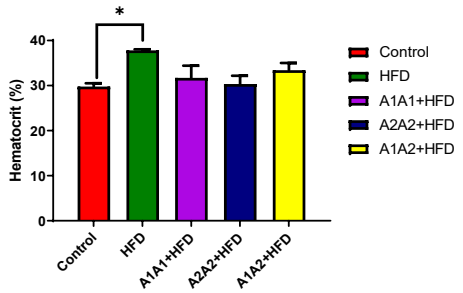

Figure1 (d)

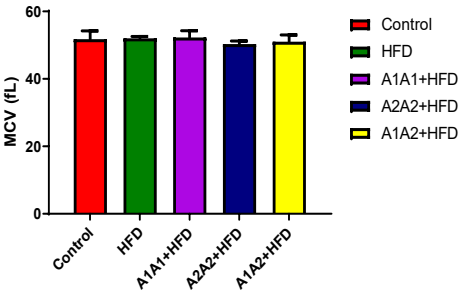

Figure1 (e)

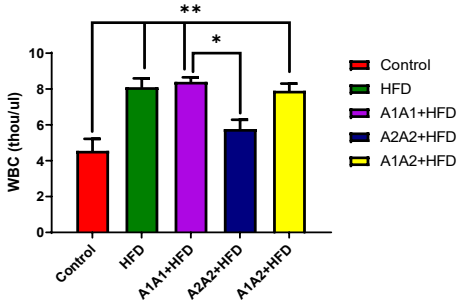

Figure1 (f)

Figure1: Graphical representation of changes in haematological parameters across different diet groups. 1a: Blood glucose; 1b: Hemoglobin (Hb); 1c: Hematocrit (HCT); 1d: Red Blood Cell (RBC); 1e: Mean Corpuscle Volume (MCV); 1f: White Blood Cell (WBC) count. *Results are shown in Mean + S.E.M. The results were considered statistically significant, *=p<0.05, **=P<0.01,***=P<0.001 and ****=p<0.0001.*

count in HFD+A1A1 group aligns with other finding signs of inflammation were observed on feeding of A1 milk in animal (Barnett et al., 2014; Haq et al.,2014;Kaminski et al., 2011; Yadav et al., 2020) as well human subjects (Jianqin et al., 2016). Evidence suggests that oxidative stress promotes the production of free radicals, which can influence the composition of circulating blood cells, WBCs. The significant reduction in WBC count observed in the HFD+A2A2 diet group indicates a protective role of the antioxidant defense system in mitigating oxidative stress-induced inflammation.

The C57BL/6J mice are reliable model for studying diet-induced alterations in hematological parameters. Significant changes were observed in mice fed a high-fat diet compared to the control group for nearly all parameters, except for mean corpuscular volume. Furthermore, feeding obese mice an A1A1 milk powder-based diet resulted in a further increase in glucose levels and white blood cell counts, indicating a potential pro-inflammatory response associated with the A1A1 beta-casein genotype. However, among the milk powder-based diet groups, notable differences between the A1A1 and A2A2 groups were limited to glucose levels and WBC counts, with no significant alterations in RBC related parameters. This is a preliminary study, and further investigation into additional aspects/parameters is necessary to draw more definitive conclusions.

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Ethical approval

"Animal trials were carried out in compliance with the Institutional Animal Ethics Committee (IAEC) protocols at the ICAR-NDRI."

DECLARATION

"The authors declare no competing interests."

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