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Physiological health and metabolic responses to glycerol supplementation in early-lactating crossbred cows: Haematological, immunomodulatory, and serum biochemical perspectives

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Abstract

Purified crude glycerol (PCG), a co-product of biodiesel manufacture, is widely investigated as a glycogenic feed supplement in dairy cattle, yet its influence on the internal physiological status of the animal has received comparatively less attention than its effects on intake and milk yield. This study examined the haematological, immunological, and serum biochemical responses of early-lactating Holstein-Friesian crossbred cows to dietary PCG offered in liquid or block-lick form. Twelve lactating cows were allotted to a control group fed only the basal diet and two treatment groups receiving PCG either as a liquid drench (360 g/day) mixed with concentrate or via free-choice access to a glycerol block lick (yielding an average PCG intake of 210 g/day), over a six-month feeding period. Blood was drawn before and after the feeding trial for haematological and serum biochemical analysis. Haemoglobin concentration rose significantly after the trial in all groups, with the rise more pronounced in glycerol-fed cows, indicating improved oxygen-carrying capacity. Total leukocyte counts and monocyte percentages increased significantly in the glycerol-supplemented groups relative to the control, pointing to an immunomodulatory response consistent with improved energy availability for immune cell turnover. Serum albumin concentration increased significantly after supplementation while globulin declined, together producing a significant improvement in the albumin-to-globulin ratio, indicative of enhanced hepatic protein synthesis. Blood glucose concentration rose markedly in the glycerol-fed groups, confirming the glycogenic action of glycerol through ruminal propionate production and hepatic gluconeogenesis. Importantly, hepatic enzymes and renal indices remained statistically unchanged across groups, indicating that glycerol supplementation did not compromise liver or kidney function. These findings support purified crude glycerol as a physiologically safe, glycogenic and immune-supportive dietary supplement for early-lactating crossbred cows.

Keywords: Glycerol, haematology, immunomodulation, serum biochemistry, glycogenic, crossbred cows

Introduction

The growing global demand for biofuels has increased the production of biodiesel and, with it, the availability of glycerol (1, 2, 3-propanetriol) as a low-cost co-product (Donkin, 2008) [7]. Because glycerol is also generated endogenously in the animal body during lipolysis of triglycerides, it has long been regarded as a physiologically compatible feed ingredient, and the United States Food and Drug Administration has recognised it as a safe feed additive for livestock. Purified and crude forms of glycerol have since been evaluated extensively as an energy and glycogenic supplement in dairy cattle diets, most commonly with an emphasis on dry matter intake, nutrient digestibility, and milk yield or composition (Ezequiel *et al.*, 2015; Kholif, 2019; Kupczynski *et al.*, 2020) [8, 14, 15].

Comparatively less emphasis has been placed on how glycerol supplementation shapes the internal physiological state of the animal, even though the haematological and biochemical profile of a dairy cow provides one of the most sensitive early indicators of nutritional adequacy, metabolic adaptation, and organ health. Glycerol that escapes rumen fermentation is rapidly absorbed and, together with the propionate generated from its fermentation, is channelled into hepatic gluconeogenesis, making it a more direct glycogenic precursor than several conventional feed additives such as propylene glycol (DeFrain *et al.*, 2004; Khalili *et al.*, 1997; Loksha and Srinivas, 2019) [6, 13, 16].

This glycogenic action is expected to influence not only circulating glucose but also downstream processes that depend on adequate glucose and energy supply, including erythropoiesis, leukocyte turnover, and hepatic protein synthesis. At the same time, because glycerol and its associated propionate load are processed principally by the liver and excreted partly via the kidney, any adverse loading effect on hepatic or renal function would be expected to manifest as altered liver enzyme or renal clearance markers. Negative energy balance in early lactation is already known to compromise immune competence in dairy cows, since both milk synthesis and immune cell activity compete for a limited circulating glucose pool (Habel and Sundrum, 2023) [9]. A glycogenic supplement that reliably raises blood glucose without imposing hepatic or renal strain could therefore carry a secondary, immunological benefit beyond its role as an energy source. However, direct evidence connecting dietary glycerol to haematological indices, leukocyte and monocyte responses, and the albumin-to-globulin balance in crossbred dairy cows remains limited. The present study was therefore designed to shift the analytical focus away from intake and production parameters and towards the internal physiological and metabolic response of early-lactating Holstein-Friesian crossbred cows to purified crude glycerol (PCG) offered either as a liquid drench or as a compressed block lick. Specifically, the haematological profile, indices of immune cell response, serum protein and albumin-to-globulin status, glycaemic response, and the safety of hepatic and renal function were evaluated to establish whether PCG supplementation supports, rather than compromises, the physiological health of the lactating cow.

Materials and Methods

Location and animals

The trial was conducted at the Livestock Research Centre, Southern Regional Station, ICAR-National Dairy Research Institute, Bengaluru, Karnataka, India (12.947014° N, 77.607679° E; elevation 921 m above mean sea level), an area with a tropical Aw (Savanna, wet) climate under the Köppen-Geiger classification. Twelve lactating Holstein-Friesian crossbred cows (body weight $\sim 420 \pm 21.50$ kg) were randomly allotted to three groups of four animals each, using milk yield as the randomisation variable and body weight as a covariate. Cows were housed in individual, well-ventilated stalls with free access to water and were dewormed with fenbendazole prior to the start of the trial. The experiment was approved by the Institutional Animal Ethics Committee, SRS, ICAR-NDRI, Bengaluru (Approval No. CPCSEA/IAEC/LA/SRS-ICAR-NDRI-2019/NO.15), and animals were managed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Dietary treatments

All cows received 12 kg/day mixed green fodder, ragi straw ad libitum, and 5 kg/day of a concentrate supplement formulated as per NRC (2001) recommendations [21]. The control group (CG) received only this basal diet. Treatment group T₁ additionally received 300 mL/day (360 g/day) of liquid PCG mixed into the concentrate. Treatment group T₂ was offered a 2 kg glycerol block lick (formulated with 50% PCG, mineral mixture, wheat bran as a filler, binder, and

common salt) for free-choice intake. This resulted in an average block consumption of approximately 420 g/day, equating to an actual PCG intake of 210 g/day. Feeding was continued for six months.

Blood collection and analysis

Blood and plasma samples were collected from the jugular vein at the beginning and end of the six-month feeding trial. Haematological indices, including haemoglobin, total and differential leukocyte counts, red blood cell counts, platelets, and packed cell volume, were determined using an automated haemoanalyser (Mindray BC-2800 Vet, Mindray Medical International Ltd., Shenzhen, China). Serum biochemical analytes, viz. total protein, albumin, blood urea nitrogen, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, triglycerides, glucose, and creatinine, were assayed using commercial diagnostic kits (Span Diagnostics Ltd., India) as per the manufacturer's protocol, with absorbance measured on a UV spectrophotometer (LMSP-UV1200, Labman Scientific Instruments Pvt. Ltd., Chennai, India). Globulin concentration and the albumin-to-globulin (A/G) ratio were derived from total protein and albumin values.

Statistical analysis

Data were analysed in a completely randomised design using the model $Y_{ij} = \mu + T_i + B_j + e_{ij}$, where T_i is the treatment effect, B_j is the replication effect, and e_{ij} is the random error. Group means were compared by Duncan's multiple range test, with significance declared at $p < 0.05$ unless stated otherwise. Paired-sample t-tests were used to compare blood and plasma values before and after the trial within each group. All statistical analyses were carried out using SPSS (version 20.0, IBM India Pvt. Ltd.) and the online statistical package VassarStats.

Results and Discussion

Haematological response to glycerol supplementation

Haemoglobin concentration increased significantly after the trial across all three groups, rising from a pre-trial mean of 7.15-7.27 g% to 7.72-8.07 g% post-trial ($P = 0.01$), with the numerically highest post-trial value recorded in the liquid-glycerol group, T₁ (Table 1). Because haemoglobin synthesis and erythropoiesis are energy-dependent processes, the somewhat greater rise observed in glycerol-supplemented cows is consistent with improved energy status arising from the additional glycogenic substrate, in agreement with reports that adequate glucose and propionate supply support erythropoietic activity in ruminants receiving glycogenic feed additives (DeFrain *et al.*, 2004; Van Soest *et al.*, 2024) [6, 20]. Red blood cell count, platelet count, and packed cell volume did not differ significantly among groups, indicating that PCG supplementation, in either liquid or block form, did not disturb the basic erythroid compartment or provoke any haemoconcentration or dilution effect.

Leukocyte and monocyte response: evidence of immunomodulation

Total leukocyte count increased significantly ($P = 0.03$) after the trial, with the highest post-trial value in T₁ ($13.02 \times 10^3/\mu\text{L}$), followed by T₂ ($12.10 \times 10^3/\mu\text{L}$) and CG ($10.22 \times 10^3/\mu\text{L}$) (Table 1). Monocyte percentage followed a similar

pattern, rising significantly ($P = 0.05$) to 1.00% in both T_1 and T_2 against 0.82% in the control group. Lymphocyte and granulocyte percentages did not differ significantly among groups, suggesting that the response was concentrated in the monocyte lineage rather than a generalised leukocytosis. Because circulating immune cells rely heavily on glucose for proliferation and phagocytic activity, and because negative energy balance is known to restrict the glucose available for immune function in dairy cows (Habel and Sundrum, 2023) ^[9], the higher leukocyte and monocyte values in glycerol-fed cows are consistent with an improved capacity to support immune cell turnover once additional glycogenic substrate becomes available. A comparable stimulatory effect of glycogenic dietary supplementation on circulating leukocyte populations has been reported in early-lactation cows receiving dietary glycerin (Chung *et al.*, 2007; Van Soest *et al.*, 2024) ^[5, 20], reinforcing the interpretation that the monocyte and leukocyte responses observed here reflect an immunomodulatory, rather than an inflammatory or pathological, adaptation. No clinical signs of infection or inflammation were observed in any animal during the trial, further supporting a physiological rather than pathological basis for the elevated cell counts.

Serum protein status: Albumin, globulin, and the albumin-to-globulin ratio

Total serum protein remained statistically similar across groups before and after the trial ($p > 0.05$), indicating that overall protein status was preserved (Table 2). However, serum albumin concentration increased significantly ($P = 0.04$) after supplementation, with the greatest rise recorded in T_2 (2.90 to 3.17 g/dL), followed by T_1 (2.75 to 3.02 g/dL) and CG (2.80 to 3.12 g/dL). Globulin concentration declined significantly ($P = 0.03$) in CG and T_2 but changed only marginally in T_1 , and this divergent pattern produced a significant improvement in the albumin-to-globulin ratio in the glycerol-supplemented groups ($P = 0.02$), rising from a pre-trial range of 0.61-0.64 to a post-trial range of 0.72-0.82. Because albumin is synthesised exclusively in the liver and its production is sensitive to the hepatic energy and amino-acid supply, the greater albumin gain in glycerol-fed cows is consistent with enhanced hepatic protein synthetic capacity supported by the additional glycogenic and propionate-derived carbon skeletons made available through glycerol fermentation (Ezequiel *et al.*, 2015; Kass *et al.*, 2013) ^[8, 10]. Comparable improvements in the albumin-to-globulin balance have been associated with better metabolic and health status in dairy cows in previous reports (Bobbo *et al.*, 2017; Kholif, 2019) ^[3, 14], where a higher A/G ratio is generally interpreted as reflecting reduced inflammatory or antigenic challenge relative to the animal's synthetic capacity for albumin. The A/G values recorded after supplementation in the present study remained within, or close to, ranges reported for clinically healthy lactating dairy cows (Bobbo *et al.*, 2017) ^[3], indicating that the shift represented a favourable adjustment within the normal physiological range rather than a pathological deviation.

Glycaemic response: validating the glycogenic role of glycerol

Blood glucose concentration provided the clearest confirmation of the glycogenic action of dietary glycerol. Glucose rose from a pre-trial value of 32.12 mg/dL to 81.85 mg/dL in T_1 and from 42.87 mg/dL to 69.37 mg/dL in T_2 , whereas the control group showed no material change (52.70 to 52.23 mg/dL) ($P = 0.01$, Table 2). This pattern is consistent with the established metabolic fate of dietary glycerol in ruminants, in which glycerol is rapidly fermented in the rumen to propionate, and any glycerol escaping ruminal fermentation is absorbed directly and delivered to the liver, where both propionate and glycerol itself are efficient substrates for hepatic gluconeogenesis (DeFrain *et al.*, 2004; Kupczynski *et al.*, 2020; Omazic *et al.*, 2013) ^[6, 15, 17]. The comparatively larger glucose response in the liquid-glycerol group (T_1) relative to the block-lick group (T_2) likely reflects both the physical form of delivery and the actual volume consumed. Cows in T_1 ingested an absolute dose of 360 g/day of PCG, whereas the free-choice block licking in T_2 resulted in a lower actual PCG intake of exactly 210 g/day. This dose-dependent variance in circulating glucose conversion is a possibility also noted in meta-analytic evaluations of glycerine supplementation across cattle studies (Syahniar *et al.*, 2021) ^[18]. Triglyceride concentration increased only modestly and non-significantly in the supplemented groups ($p > 0.05$), indicating that the additional glycogenic flux was not accompanied by an adverse shift towards lipogenesis or lipid accumulation.

Hepatic and renal safety markers

Despite the substantial glycaemic shift, none of the hepatic or renal indices measured showed a significant change with glycerol supplementation. Alanine aminotransferase (34.57-47.65 IU/L) and aspartate aminotransferase (100.49-127.57 IU/L) did not differ significantly among groups after the trial ($p > 0.05$), nor did the AST:ALT ratio, indicating that glycerol supplementation, at the dose and duration employed, did not impose detectable hepatocellular stress. Similarly, blood urea nitrogen (17.27-18.87 mg/dL) and serum creatinine (1.02-1.15 mg/dL) remained statistically unchanged across groups, suggesting that renal filtration and clearance function were not adversely affected by the additional metabolic load associated with glycerol and propionate handling. These observations are consistent with previous reports in which crude or purified glycerol supplementation, even at levels considerably higher than that used in the present study, did not produce clinically relevant elevations in liver enzymes or renal markers in dairy cattle (Ezequiel *et al.*, 2015; Kass *et al.*, 2013; Van Cleef *et al.*, 2016) ^[8, 10, 19]. Taken together with the favourable haematological, immune, and protein responses described above, the absence of any adverse hepatic or renal signal reinforces that purified crude glycerol, delivered either as a liquid drench or as a block lick, can be regarded as a physiologically safe glycogenic supplement for early-lactating crossbred cows.

Table 1: Haematological variables of crossbred cows fed control (CG) or glycerol-supplemented (T₁, T₂) diets, before and after the six-month feeding trial

Parameter	CG (Before)	T ₁ (Before)	T ₂ (Before)	CG (After)	T ₁ (After)	T ₂ (After)	SEM	P value
Haemoglobin (g%)	7.15	7.27	7.17	7.85	8.07	7.72	0.07	0.01
WBC ($\times 10^3/\mu\text{L}$)	9.75	13.37	12.17	10.22a	13.02ab	12.10b	0.40	0.03
RBC ($\times 10^{12}/\mu\text{L}$)	4.90	5.14	5.30	5.77	5.48	5.40	0.18	0.27
Platelets ($\times 10^3/\mu\text{L}$)	424	478	454	339	416	392	24.00	0.21
PCV (%)	23.90	24.50	23.60	25.80	26.50	26.20	0.45	0.36
Lymphocytes (%)	4.70	4.65	5.05	5.20	5.02	5.40	0.20	0.29
Granulocytes (%)	4.35	7.42	6.25	4.47	5.35	5.57	0.30	0.25
Monocytes (%)	0.70	1.12	0.87	0.82a	1.00b	1.00b	0.04	0.05

Values bearing different superscripts (a, b) within the same row differ significantly ($p < 0.05$). CG = control; T₁ = liquid glycerol; T₂ = glycerol block lick; SEM = standard error of mean.

Table 2: Serum biochemical variables of crossbred cows fed control (CG) or glycerol-supplemented (T₁, T₂) diets, before and after the six-month feeding trial

Parameter	CG (Before)	T ₁ (Before)	T ₂ (Before)	CG (After)	T ₁ (After)	T ₂ (After)	SEM	P value
Total protein (g/dL)	7.37	7.20	7.42	7.05	7.20	7.05	0.09	0.42
Albumin (g/dL)	2.80	2.75	2.90	3.12	3.02	3.17	0.07	0.04
Globulin (g/dL)	4.57	4.45	4.52	3.93	4.18	3.88	0.08	0.03
A/G ratio	0.61	0.62	0.64	0.79	0.72	0.82	0.04	0.02
Glucose (mg/dL)	52.70	32.12	42.87	52.23	81.85	69.37	5.00	0.01
Triglycerides (mg/dL)	10.15	9.85	10.55	10.30	12.00	10.97	0.50	0.12
ALT (IU/L)	46.80	38.55	49.20	41.62	34.57	47.65	4.00	0.28
AST (IU/L)	104.00	99.20	108.35	107.21	100.49	127.57	6.00	0.19
AST:ALT	2.24	2.57	2.20	2.68	2.89	2.67	0.15	0.21
BUN (mg/dL)	19.30	23.75	19.75	18.87	17.27	17.90	1.80	0.16
Creatinine (mg/dL)	0.95	1.07	1.00	1.02	1.12	1.15	0.05	0.23

A/G ratio = albumin-to-globulin ratio; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BUN = blood urea nitrogen; CG = control; T₁ = liquid glycerol; T₂ = glycerol block lick; SEM = standard error of

Conclusion

Dietary supplementation with purified crude glycerol, offered either as a liquid drench or as a compressed block lick, produced a coordinated set of favourable internal physiological responses in early-lactating Holstein-Friesian crossbred cows. Haemoglobin concentration and total leukocyte and monocyte counts increased significantly, indicating improved oxygen-carrying capacity and enhanced immune cell availability. Serum albumin concentration rose while globulin declined, yielding a significantly improved albumin-to-globulin ratio suggestive of better hepatic protein synthetic status. Blood glucose concentration increased markedly in glycerol-fed cows, directly confirming the glycogenic role of glycerol through ruminal propionate production and hepatic gluconeogenesis. Critically, hepatic enzymes and renal function markers remained unaffected, confirming that these metabolic and haematological benefits were achieved without compromising liver or kidney health. Purified crude glycerol can therefore be recommended as a physiologically safe and metabolically beneficial glycogenic supplement for early-lactating crossbred dairy cows, with the liquid form showing a marginally stronger glycaemic response than the block-lick form.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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