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Research Article

Effect of urea adulteration on the nitrogen distribution of cow and buffalo milk

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Abstract

Adulteration of milk and milk products is one of the troublesome food safety issue of Indian subcontinent. Nitrogen containing substances like urea, ammonium sulphate, melamine, etc. are some of the prevalent adulterants in milk and milk products. In this study, urea was added in both cow and buffalo milk at different levels viz. 200, 300 and 400 mg/100 mL milk. Nitrogen fractions of milk were analysed using Kjeldahl method to know the nitrogen distribution in milk as a function of extraneous urea addition. Upon adulteration of cow, buffalo and mixed milks by adjusting their total nitrogen with 0, 200, 300 and 400 mg of ureaper 100 mL their respective mean values of true protein nitrogen (TPN) were 0.532, 0.439, 0.408 and 0.373 per cent in cow milk; 0.575, 0.455, 0.384 and 0.374 per cent in buffalo milk and 0.572, 0.483, 0.417 and 0.376 per cent in mixed milk. The respective mean values of casein nitrogen (CN) were 0.424, 0.349, 0.327 and 0.281 per cent in cow milk; 0.472, 0.398, 0.321 and 0.303 per cent in buffalo milk and 0.477, 0.410, 0.339 and 0.303 per cent in mixed milk. The respective mean values of non-protein nitrogen (NPN) were 0.039, 0.120, 0.153 and 0.182 per cent in cow milk; 0.040, 0.149, 0.190 and 0.226 per cent in buffalo milk and 0.039, 0.110, 0.140 and 0.171 per cent in mixed milk. However, no significant differences were observed in changes in whey protein nitrogen (WPN) content in all the three types of milk.

Keywords: Adulteration, Milk, Non-protein nitrogen, Urea

Highlights

- Data on nitrogen distribution, both in pure and adulterated milk, was generated
- Impact of urea adulteration on nitrogen fractions of milk was assessed.
- Measuring fractions like true protein nitrogen, casein nitrogen and non-protein nitrogen can help detect urea adulteration in milk
- Urea adulteration leads to significant decrease in the TPN and CN.
- NPN fraction is significantly increased upon urea adulteration

INTRODUCTION

Nitrogenous substances like urea, ammonium sulphate, melamine, etc. are widely used as an extraneous source to falsely raise the protein and solids-not-fat (SNF) content of milk. These extraneous water-soluble nitrogenous compounds in milk increases the total nitrogen content giving false impression of high protein content in milk. Therefore, they can be widely used as an adulterant in milk (Finete et al., 2013). As per the reports of Garg and Mulla (2024), urea is one of the prevalent adulterants found in milk sold in many Indian cities. The urea adulteration in milk can have implications on nutritional value of milk and may cause health hazards (Ibrahim et al., 2025). Arun Kommadath et al. (2001) in his study on mice has indicated urea fed at 7.3 mg/kg/day caused kidney and liver damage. HPLC, infrared and Raman spectroscopy, wet chemistry-based para-dimethylaminobenzaldehyde (DMAB), enzyme-based, and biosensor-based methods are conventionally used for the detection or determination of urea in milk (Savaliya et al., 2022; Shalileh et al., 2023). As urea is an inherent component of milk, Food Safety and Standards Authority of India [FSSAI] (2011) has set the limit of total urea content in milk as 70 mg/100 mL.

Urea constitutes about 50% to the total NPN content of milk. Adulteration of milk with such substances is prevalent as Kjeldahl method for protein analysis cannot distinguish between nitrogen from protein and non-protein sources. This results in incorrectly high protein in products containing non-protein nitrogen sources like urea, melamine and causes an economic gain out of its addition. Particularly, change in NPN fraction of milk can be an indication to ascertain the adulteration of milk with

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such extraneous substance. Hence, a study was undertaken to understand the impact of urea adulteration on various nitrogen fractions of milk.

MATERIALS AND METHODS

Collection of cow and buffalo milk: Pooled samples of cow and buffalo milk were procured from animal farms maintained at Anand Agricultural University, Anand, Gujarat (India) and commercial dairy plants of Anand city. Mixed milk was prepared by mixing cow and buffalo milk (50:50).

Preparation of solution containing nitrogenous substances: The solution containing 1% urea was prepared at laboratory level. Such solution also contained substances like vegetable oil, maltodextrin, sucrose and salts to emulate milk like appearance. The targeted level of NPN content in milk was achieved by calculated admixing of the prepared solution in milk (cow/buffalo) as mentioned in the Table 1.

Table 1. Preparation of adulterated milk samples containing urea

Pure milk (mL)	Solution containing Urea (mL)	Urea supplied by the solution (mg/100 mL)
100	0	0
80	20	200
70	30	300
60	40	400

Estimation of nitrogen fractions in milk: Total Nitrogen (TN), Non-casein Nitrogen (NCN) and Non-protein Nitrogen (NPN) fractionsin milk were determined by Micro Kjeldahl Methods (Association of Official Analytical Chemists [AOAC], 2010) using block digester for digestion of sample and automatic nitrogen distillation system for distillation. The True Protein Nitrogen (TPN) and Whey Protein Nitrogen (WPN) were derived by subtracting NPN from TN and NCN respectively.

Statistical analysis of the data

The data of various parameters were analyzed using Analysis of Variance (ANOVA). Difference between treatments was evaluated at 5% level of significance.

RESULTS

Various nitrogen fractions of milk were determined and are discussed below.

Nitrogen distribution in different types of milk: In this study, 24 samples each of cow, buffalo and mixed milk were collected and analysed at an interval of 15 days for the TN, NCN and NPN using the Kjeldahl method. TPN, CN and WPN were derived from above analyzed parameters. The results of the nitrogen distribution in milk are presented in Table 2.

Table 2. Nitrogen distribution in different types of milk

Types of milk	Nitrogen fractions (%) TN	TPN	CN	WPN	NPN
Cow Milk	0.5287^{a}	0.4898^{a}	0.4041^{a}	0.0857^{a}	0.0416
Buffalo Milk	0.6432^{b}	0.6015^{b}	0.5100^{b}	0.0915^{b}	0.0389
Mixed Milk	0.5410^{a}	0.5027^{a}	0.4216^{a}	0.0811^{a}	0.0383
SEm.	0.007	0.0068	0.0059	0.0029	0.0020
CD(P<0.05)	0.02	0.019	0.085	0.0082	NS
CV%	6.40	6.55	6.77	17.38	26.82

*Values are means of 24 samples.

**Superscripts a and b indicate significant difference between treatments.

The average TN, TPN, CN, WPN and NPN in cow milk were found to be 0.5287, 0.4898, 0.4041, 0.0857 and 0.0416 per cent, whereas, in case of buffalo milk these values were 0.6432, 0.6015, 0.5100, 0.0915 and 0.0389 per cent, respectively. In mixed milk, the values obtained for the same parameters were 0.5410, 0.5027, 0.4216 and 0.0383 per cent respectively.

Table 3. Effect of urea adulteration on total nitrogen of cow and buffalo milk

Added urea in milk (mg/100 mL)	Cow Milk TN (%) Range	Mean*	Buffalo Milk TN (%) Range	Mean*
Pure milk	0.513-0.624	0.571	0.539-0.691	0.616
200	0.529-0.615	0.559	0.552-0.652	0.604
300	0.523-0.606	0.562	0.547-0.606	0.574
400	0.523-0.595	0.555	0.552-0.628	0.599
SEm		0.015	0.017	
CD (P<0.05)		NS	NS	
CV%		5.98	6.42	

* The values are mean of 5 samples.

Effect of urea adulteration on nitrogen fractions: The effect of addition of urea on TN, TPN, CN, WPN and NPN of both cow and

Table 4. Effect of urea adulteration on true protein nitrogen of cow and buffalo milk

Added urea in milk (mg/ 100 mL)	Cow Milk TPN (%)			Buffalo Milk TPN (%)		
	Range	Mean*	% of TN	Range	Mean*	% of TN
0	0.476-0.581	0.532^{a}	93.17	0.504-0.651	0.575^{a}	93.34
200	0.388-0.487	0.439^{b}	78.53	0.411-0.478	0.455^{b}	75.33
300	0.355-0.448	0.408^{bc}	72.60	0.358-0.412	0.384^{c}	66.90
400	0.311-0.431	0.373^{c}	67.20	0.325-0.414	0.374^{c}	62.44
SEm			0.019		0.017	
CD (P<0.05)			0.056		0.050	
CV%			9.52	8.27		

* The values are mean of 5 samples.
**Superscripts a, b and c indicate significant difference between treatments.

Table 5. Effect of urea adulteration on casein nitrogen of cow and buffalo milk

Added urea in milk (mg/ 100 mL)	Cow Milk CN (%)			Buffalo Milk CN (%)		
	Range	Mean*	% of TN	Range	Mean*	% of TN
0	0.375-0.467	0.424^{a}	74.26	0.379-0.539	0.472^{a}	76.62
200	0.310-0.393	0.349^{b}	62.43	0.364-0.435	0.398^{b}	65.89
300	0.274-0.377	0.327^{bc}	58.19	0.271-0.359	0.321^{c}	55.92
400	0.225-0.350	0.281^{c}	50.63	0.277-0.327	0.303^{c}	50.58
SEm			0.019		0.018	
CD (P<0.05)			0.058		0.053	
CV%			12.46		10.66	

* The values are mean of 5 samples.
**Superscripts a, b and c indicate significant difference between treatments.

buffalo milk was evaluated. The results obtained are shown in the below given sections.

Effect of urea adulteration on total nitrogen of cow and buffalo milk: The mean value of TN of pure and urea adulterated cow and buffalo milk is shown in Table 3.

The mean value of TN was found to be 0.571, 0.559, 0.562 and 0.555 per cent in the cow milk containing 0, 200, 300 and 400 mg/100 mL of urea, respectively. Similarly, the values observed in buffalo milk were 0.616, 0.604, 0.574, and 0.599 per cent under the same urea concentrations.

Effect of urea adulteration on true protein nitrogen of cow and buffalo milk: The TPN was obtained by deducting the NPN from the TN. The values obtained for pure and urea adulterated cow and buffalo milk is shown in Table 4.

The mean value of per cent TPN for pure cow milk was observed to be 0.532. When milk was adulterated with 200, 300 and 400 mg urea per 100 mL; per cent TPN decreased to 0.439, 0.405 and 0.373, respectively. The TPN content of pure cow milk and urea adulterated cow milk differed significantly. Further, in pure cow milk TPN amounted to 93.17 per cent in TN and decreased to 78.53, 72.60 and 67.20 per cent, respectively in the milk samples adulterated with 200, 300 and 400 mg urea per 100 mL. Thus, in comparison to pure cow milk, percentage of TPN in TN decreased by 14.64, 20.57 and 25.97 per cent respectively.

The mean value of per cent TPN for pure buffalo milk was observed to be 0.575. When milk was adulterated with 200, 300 and 400 mg urea per 100 mL; per cent TPN found to be decreased to the level of 0.455, 0.384 and 0.374, respectively. TPN content showed decreasing trend as amount of urea concentration in adulterated buffalo milk increased. Significant difference was observed between the TPN content of pure buffalo milk and all urea adulterated buffalo milk samples. Further, the TPN of pure buffalo milk contributed 93.34 per cent to TN. When the milk was adulterated with 200, 300 and 400 mg urea per 100 mL; the amount of TPN in TN decreased to 75.33, 66.90 and 62.44 per cent respectively. Thus, in comparison to pure buffalo milk, percentage of TPN in

Table 6. Effect of urea adulteration on whey protein nitrogen of cow and buffalo milk

Added urea in milk (mg/ 100 mL)	Cow Milk WPN (%)			Buffalo Milk WPN (%)		
	Range	Mean*	% of TN	Range	Mean*	% of TN
0	0.086-0.169	0.109	19.08	0.081-0.125	0.103	16.72
200	0.057-0.134	0.090	16.10	0.043-0.075	0.057	9.44
300	0.070-0.107	0.081	14.41	0.041-0.118	0.064	11.15
400	0.081-0.119	0.092	16.57	0.041-0.124	0.071	11.85
SEm			0.011		0.012	
CD (P<0.05)			NS		NS	
CV%			26.83		35.94	

* The values are mean of 5 samples.

TN decreased by 18.01, 26.44 and 30.90 per cent respectively. The decrease in the TPN was linearly related with the levels of urea adulteration in milk.

Effect of urea adulteration on casein nitrogen of cow and buffalo milk: The mean value of CN of pure and urea adulterated cow and buffalo milk is shown in the Table 5.

The mean value of per cent CN for pure cow milk was observed to be 0.424. When milk was adulterated with 200, 300 and 400 mg urea per 100 mL; per cent CN decreased to 0.349, 0.327 and 0.281 respectively. The CN content of pure cow milk and urea adulterated cow milk differed significantly. Further, the CN accounted for 74.26 per cent in TN of pure cow milk. When the milk was adulterated with 200, 300 and 400 mg urea per 100 mL; the amount of CN in TN decreased to 62.43, 58.19 and 50.63 per cent respectively. Thus, in comparison to pure cow milk, percentage of CN in TN decreased by 11.83, 16.07 and 23.63 per cent respectively. The CN content decreased linearly with the levels of urea adulteration in milk. These values of CN in urea adulterated milk samples indicated a noticeable effect on CN fraction of milk.

The mean value of per cent CN for pure buffalo milk was observed to be 0.472. When milk was adulterated with 200, 300 and 400 mg urea per 100 mL; per cent CN decreased to 0.398, 0.321 and 0.303 respectively. The CN content of pure buffalo milk and urea adulterated buffalo milk differed significantly. Further, in pure buffalo milk, the CN amounted to 76.62 per cent in TN. When the milk was adulterated with 200, 300 and 400 mg urea per 100 mL; the amount of CN in TN decreased to 65.89, 55.92 and 50.58 per cent respectively. Thus, in comparison to pure buffalo milk, percentage of CN in TN decreased by 10.73, 20.70 and 26.04 per cent respectively.

Effect of urea adulteration on whey protein nitrogen of cow and buffalo milk: The mean value of WPN of pure and urea adulterated cow and buffalo milk is shown in Table 6.

The mean value of per cent WPN for pure cow milk was observed to be 0.109. When milk was adulterated with 200, 300 and 400 mg urea per 100 mL; per cent WPN obtained were 0.090, 0.081 and 0.092 respectively. Further, in pure cow milk WPN amounted 19.08 per cent in TN. When the milk was adulterated with 200, 300 and 400 mg urea per 100 mL; the amounts of WPN in TN observed were 16.10, 14.41 and 16.57 per cent respectively. This showed inconsistent interaction of urea with the WPN fraction of milk. The difference between the WPN fraction of pure and urea adulterated milk was found to be non-significant.

The mean value of per cent WPN for pure buffalo milk was observed to be 0.103. When milk was adulterated with 200, 300 and 400 mg urea per 100 mL; per cent WPN obtained were 0.057, 0.064 and 0.071 respectively. Further, in pure milk WPN amounted 16.72 per cent in TN. When the milk was adulterated with 200, 300 and 400 mg urea per 100 mL; the amounts of WPN in TN observed were 9.44, 11.15 and 11.85 per cent respectively. This showed inconsistent interaction of urea with the WPN fraction of milk. The difference between the WPN fraction of pure and urea adulterated milk was found to be non-significant.

Effect of urea adulteration on non-protein nitrogen of cow and buffalo milk: The mean value of NPN of pure and urea adulterated cow and buffalo milk is shown in Table 7.

The mean value of per cent NPN for pure cow milk was observed to be 0.039. When milk was adulterated with 200, 300 and 400 mg urea per 100 mL; per cent respectively. These values are in alignment with results we have obtained in unadulterated cow and buffalo milk in our study.

Effect of urea adulteration on true protein nitrogen of cow and buffalo milk: The decrease in the TPN was linear with the levels of urea adulteration in both cow and buffalo milk. These values of TPN in urea adulterated milk samples indicated a noticeable effect on TPN fraction of milk. The lowest level of urea adulteration *i.e.* 200 mg/100 mL milk led to significant difference in TPN from the unadulterated milk of both cow and buffalo. This is plausible as one of the largest (~50 per cent) and most stable NPN part is formed by milk urea (Ruska & Jonkus, 2014); therefore urea used as adulterant would decrease TPN content.

The mean value of TPN obtained in case of unadulterated cow and buffalo milk samples was in line with reported values of TPN by Pandya and Khan (2006). However, this value was lower than reported values by Ahmed et al. (2013), Kapadia et al. (2016), and Rafiq et al. (2016).

Effect of urea adulteration on casein nitrogen of cow and buffalo milk: The CN significantly differed even with lowest level of urea addition *i.e* 200 mg/100 mL in both cow and buffalo milk. Further, a concomitant decrease in the CN content of adulterated samples of both cow and buffalo milk was noted in line with levels of urea adulteration. Hence, values of CN in urea adulterated milk samples indicated a noticeable effect on CN fraction of milk. Adulteration of milk with spurious milk, particularly so called synthetic milk can lead to lowering of this characteristic milk protein as observed in the study. Huma et al. (2018) has reported mean casein content in cow and buffalo milk as 2.58 and 2.93% (corresponding to 0.40 and 0.46% N), respectively. Ahmad et al. (2008) have reported CN (g/kg) in buffalo milk and cow milk as 34.6 and 26.1 (corresponding to 0.54 and 0.41% N), respectively.

Effect of urea adulteration on whey protein nitrogen of cow and buffalo milk: Since the adulterated cow and buffalo milk samples were prepared by mixing urea which is also soluble in water it can compensate for the possible whey protein nitrogen dilution. Thus, results obtained in the study for both the types of milk do not show any significant difference between pure milk and milk containing extraneous urea.

Effect of urea adulteration on non-protein nitrogen of cow and buffalo milk: Considering the values of the NPN for cow and buffalo milk obtained in the unadulterated (pure) milk (Table 2), the values of NPN in the adulterated milk is noticeably higher in urea adulterated milk. The NPN was the most significantly affected fractions in both urea adulterated milk samples of cow and buffalo (Table 7). The increase in NPN was in commensuration with the level of urea adulteration in both cow and buffalo milk. This is due to the fact that urea is naturally present in NPN fraction of milk. Even when milk is adulterated with extraneous urea, most of it goes in the NPN fraction. This leads to significant increase in the NPN fractions of both cow and buffalo milk (DePeters & Ferguson, 1992; De Vries et al., 2017).

Saavedra-Jiménez et al. (2019) has reported mean NPN content in Mexican Brown Swiss cattle as 0.02%, with the range being 0.01 to 0.05%. Considering the values of NPN in both cow and buffalo milk reported by various researchers (Pandya & Khan, 2006; Fox et al., 2015; Kapadiya et al., 2016), the values obtained for unadulterated cow and buffalo milk are in alignment. However, in urea-adulterated milk samples, even the lowest level of urea adulteration led to nearly a threefold increase in NPN.

Khandakar et al. (2024) in their study added various market milk powders and locally marketed milk in normal milk and analysed for various nitrogen fractions of it. They opined the potential of the nitrogen distribution of milk to detect adulterated and reconstituted market milk. Islam et al. (2024) have also analysed nitrogen distribution pattern of milk as a method for detection of presence of adulterants like water, sugar, flour in milk.

Conclusion

The True Protein Nitrogen, Casein Nitrogen and Non-protein Nitrogen fractions were proportionately decreased as urea addition increased in both cow and buffalo milk. However, Total Nitrogen and Whey Protein Nitrogen were not significantly affected by urea adulteration at all levels. A proportionate decrease in TPN and CN accompanied by a proportionate increase in NPN may indicate possible adulteration of cow and buffalo milk with urea. The nitrogen distribution pattern of normal milk is distinct, any attempt made to adulterate milk with non-protein nitrogenous substances would change the distribution of various natural nitrogen fractions of milk. This study would enable academicians and dairy processors to ascertain the quality of milk by evaluating various nitrogen fractions of milk for detection of possible adulteration with such materials.

Table 7. Effect of urea adulteration on non-protein nitrogen of cow and buffalo milk

Added urea in milk (mg/ 100 mL)	Cow Milk NPN (%)			Buffalo Milk NPN (%)		
	Range	Mean*	% of TN	Range	Mean*	% of TN
0	0.035-0.043	0.039[a]	6.83	0.035-0.044	0.040[a]	6.49
200	0.084-0.171	0.120[b]	21.46	0.140-0.174	0.149[b]	24.67
300	0.115-0.196	0.153[bc]	27.22	0.182-0.196	0.190[c]	33.10
400	0.131-0.242	0.182[c]	32.79	0.197-0.246	0.226[d]	37.73
SEm			0.016		0.005	
CD (P<0.05)			0.048		0.016	
CV%			28.88		7.88	

* The values are mean of 5 samples.

**Superscripts a, b, c and d indicate significant difference between treatments.

NPN increased to 0.120, 0.153 and 0.182 respectively. Significant difference was observed between the NPN content of pure milk and urea adulterated cow milk samples. Further, as can be observed from the Table 7, in pure cow milk NPN amounted 6.83 per cent in TN. When the milk was adulterated with 200, 300 and 400 mg urea per 100 mL; the amount of NPN in TN increased to 21.46, 27.22 and 32.79 per cent respectively. Thus, percentage of NPN in TN increased 3.14, 3.98 and 4.80 times respectively. These values of NPN in urea adulterated milk samples indicated a clear and drastic effect of urea adulteration on the NPN fraction.

The mean value of per cent NPN for pure buffalo milk was observed to be 0.040. When milk was adulterated with 200, 300 and 400 mg urea per 100 mL; per cent NPN increased to 0.149, 0.190 and 0.226 respectively. Significant difference was observed between the NPN content of pure milk and urea adulterated buffalo milk samples. Further, as can be observed from the Table 7 in pure buffalo milk NPN amounted 6.49 per cent in TN. When the milk was adulterated with 200, 300 and 400 mg urea per 100 mL; the amount of NPN in TN increased to 24.67, 33.10 and 37.73 per cent respectively. Thus, percentage of NPN in TN increased 3.80, 5.10 and 5.81 times respectively. These values of NPN in urea adulterated milk samples indicated a clear and drastic effect of urea adulteration on the NPN fraction.

DISCUSSION

Nitrogen distribution in different types of milks: The significant difference was observed between the TN, TPN, CN and WPN fractions of pure cow and buffalo milk. However no significant difference in the NPN of cow and buffalo milk was observed (Table 2). The buffalo milk contains higher protein, hence all nitrogen fractions TN, TPN, CN, WPN and NPN are relatively higher as compared to cow milk (Kapadiya et al., 2016).

Fox et al. (2015) has indicated the NPN concentration of cow milk as approximately 25 to 30 mg/100 mL of milk, contributing only 5 to 6 per cent of the total nitrogen (TN). Kapadiya et al. (2016) has reported average values of TN, NCN and NPN of cow milk as 0.547, 0.124 and 0.054 per cent respectively. Pandya and Khan (2006) have reported average values of TN, TPN, CN and NPN as 0.600, 0.573, 0.460 and 0.035 per cent respectively for buffalo milk. In terms of percent of TN, values for TPN, CN and NPN reported were 94.2, 75.6 and 5.7, respectively. Islam et al. (2014) reported TPN, CN, WPN and NPN to vary from 0.539 to 0.558, 0.413 to 0.434, 0.125 to 0.126 and 0.035 percent, respectively for Bangladeshi buffalo milk. Kapadiya et al. (2016) has reported average values of TN, NCN and NPN of buffalo milk as 0.702, 0.547, 0.140 and 0.051 per cent respectively.

It was observed that our results were in general agreement with the reported values. All nitrogen fractions, except NPN, differed significantly among types of milk.

Effect of urea adulteration on nitrogen fractions of cow and buffalo milk: No significant difference between TN content of both pure cow and buffalo milk and corresponding urea adulterated milk samples was observed. This was observed, as the adulterated samples of cow and buffalo milk was prepared by considering the total nitrogen to remain constant (Table 1). The mean values of the TN obtained in our study are lower than values reported by Ahmed et al. (2013), Kapadia et al. (2016), and Rafiq et al. (2016) for cow and buffalo milk.

Saavedra-Jiménez et al. (2019) has reported mean TN content in Mexican Brown Swiss cattle as 0.51%. Huma et al. (2018) has reported mean crude protein content in cow and buffalo milk as 3.60 and 4.20% (corresponding to 0.56 and 0.66 per cent N)

Conflict of interest: Authors have no conflict of interest in this research study.

Author's contributions: AIS: Investigation and data generation; KDA: Supervision of the study; AKJ, SCP, PDC: Preparation of manuscript; SB: Engaged in data curation.

Data availability statement: Data will be provided on request to the corresponding author.

Use of artificial intelligence tools: AI tools are not used in the study.

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