RESEARCH ARTICLE Indian Journal of Animal Research, Volume 59 Issue 2: 209-215 (February 2025)

Study of the Effects of Alfalfa Bioactive Substances and Polysaccharides on the Lactation Performance and Immune Function of Dairy Cows

Yajun Chen[1], Man Xu[1], Chuang Huang[2]

10.18805/IJAR.BF-1878

ABSTRACT

Background: The aim of this study was to investigate the effects of alfalfa polysaccharides on the lactation performance and immune function of dairy cows.

Methods: A total of 84 healthy Holstein cows were selected and randomly divided into 7 groups, including the control Group A0, the alfalfa polysaccharide groups (AP1, AP2, AP3) and the alfalfa extract groups (AE1, AE2, AE3). Each group had three replicates and each replicate had four cows. The daily rations of the dairy cows in the AP group and the AE group were supplemented with alfalfa polysaccharide powder and alfalfa extract at concentrations of 1, 2 and 3 kg/t, those in the A0 group served as a control.

Result: (1) the milk production of dairy cows in the AE groups was extremely significantly greater ($p<0.01$) than that in A0 group and AP groups. The milk protein content of cows in the AP2 and AE2 groups was significantly greater ($P<0.05$) than that in the A0 group (2) The interleukin-2 (IL-2) levels in the serum of cows in the AP2, AP3, AE2 and AE3 groups were greater than those in the A0 and AP1 groups and the IgM antibody levels in the AP group and the AE group were extremely significantly greater than those in the A0 and AP1 groups ($P<0.01$). The IgA antibody level in the serum of dairy cows in the AP2, AP3, AE2 and AE3 groups was significantly ($P<0.01$) greater than that in the A0, AP1 and AE1 groups. In summary, the alfalfa extract improved both the lactation performance and immunity of dairy cows, indicating comprehensive effects. Alfalfa polysaccharides at a concentration of 2 kg/t improved the immunity of dairy cows.

Key words: Alfalfa polysaccharides, Bioactive substances from alfalfa, Dairy cows, Immune function, Lactation performance.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a perennial high-quality forage legume planted worldwide. It is known as the ″king of legumes″ and is a high-quality feed for livestock and poultry because of its wide adaptability, high yield, high nutritional value and suitable palatability. Alfalfa is fed mainly to dairy cows in the form of green hay, grass powder and silage, which can significantly improve feed digestibility, increase the utilization rate of feed by dairy cows and reduce feeding cost (Allen *et al*., 2000; Liu *et al*., 2013). Moreover, feeding dairy cows with alfalfa hay can significantly increase the ability of rumen microbes to synthesize proteins, increase milk production and persistency during lactation and improve milk quality (Ni *et al*., 2022). But relatively high lignin content of alfalfa, restricts the digestion and absorption of nutrients by animals and leads to great waste.

Some plant extracts, such as Astragalus spp polysaccharides, are widely used in the aquaculture industry because of their natural antioxidant, proimmunity and anti-inflammatory properties (Liu *et al*., 2018). Given that the addition of antibiotics to feed is banned in China, the development and utilization of suitable natural plant extracts are particularly urgent and important (Gao *et al*., 2024). Alfalfa extract is rich in active substances such as polysaccharides, flavonoids and saponins, which not facilitate digestion and absorption but also significantly improve the growth performance and immune function of animals (Zhang *et al*., 2016; Zhan *et al*., 2018). Alfalfa polysaccharides composed of eight monosaccharides, including fucose and xylose (Gözüaçık *et al*., 2017). They have the advantages of strong activity, low toxicity and low cost. Moreover, these polysaccharides are natural free radical scavengers with antioxidant enzyme activity. Studies have shown that alfalfa polysaccharides can enhance immunity by regulating the secretion of interleukin-10 (IL-10) and nitrogen oxides (NO) and the levels of nitric oxide synthase (iNOS) in serum of hens and can enhance the immune effect of attenuated Newcastle disease vaccines

[1]Changde Vocational and Technical College, No.4253 Renmin Road, Wuling District, Changde City, Hunan Province, China.
[2]Deren Animal Husbandry Technology Co., Ltd. Xihu District, Changde City, Hunan Province, China.

Corresponding Author: Yajun Chen, Changde Vocational and Technical College, No.4253 Renmin Road, Wuling District, Changde City, Hunan Province, China. Email: chen_yajun11@163.com
ORCID: 0000-0003-0309-5247.

How to cite this article: Chen, Y., Xu, M. and Huang, C. (2025). Study of the Effects of Alfalfa Bioactive Substances and Polysaccharides on the Lactation Performance and Immune Function of Dairy Cows. Indian Journal of Animal Research. 59(2): 209-215. doi: 10.18805/IJAR.BF-1878.

Submitted: 26-08-2024 **Accepted:** 05-10-2024 **Online:** 16-10-2024

(Hisatsune *et al*., 2007; Liu *et al*., 2015) studied the antitumor activity of alfalfa polysaccharides *in vitro* and reported that alfalfa polysaccharides inhibited the activity of human breast cancer cells in a dose-dependent manner while promoting the apoptosis of breast cancer cells, exhibiting suitable antitumor activity.

However, relevant reports have focused mostly on pigs and chickens and there are no systematic reports on the effects of alfalfa extract on dairy cows. This study aimed to reveal the effects of alfalfa active components, especially alfalfa polysaccharides, on the lactation and immunity of dairy cows to provide a reference for the further use of alfalfa in the dairy industry.

MATERIALS AND METHODS

The research institution of this experiment is the Laboratory of Animal Husbandry and Veterinary Science of Changde Vocational and Technical College, as well as the second farm operated by Deren Animal Husbandry Technology Co., LTD. The experiment commenced in April 2023 and spanned a duration of 15 monthsÿincluding experimental design and data analysis. The alfalfa alcoholic extract and polysaccharide concentrate powder used in the experiments were purchased from Baicao Biote Co., Ltd., The extraction ratio was 20:1, the alfalfa polysaccharide content was 40% and starch (content ≥ 59%) was the main ingredient. The major components of the alfalfa extract are shown in Table 1. The cows used in the experiment were healthy adult Holstein cows with similar body conditions from a dairy company in Changde, Hunan, the mean parity was 3, the average age was 3.4 years, the mean milk production was 32.1 kg and the mean body weight was 695 kg, shown in Table 3.

The test period was 13 weeks, with a pilot period of 1 week and a test period of 12 weeks. A total of 84 Holstein cows were selected and randomly divided into seven groups, including the control Group A0, the alfalfa polysaccharide groups (AP1, AP2, AP3) and the alfalfa extract groups (AE1, AE2 and AE3),. Each group had three replicates and each replicate had four cows. During the experiment, the daily rations of the dairy cows in the AP group were supplemented with 1, 2, or 3 kg/t alfalfa polysaccharide powder and the daily rations of the dairy cows in the AE group were supplemented with 1, 2, or 3 kg/t alfalfa. extract and Group A0 was used as a control without adding any substance. During the experimental period, dairy cows were given daily formula rations on the farm. The ingredients and nutrient levels are shown in Table 2. After being fed under completely consistent feeding conditions for 1 week, a 12-week experiment was conducted. The ingredients and nutrient levels of the daily feed rations on the dairy farms are shown in Table 2.

During the pilot period, milk production was measured once a day and the statistical mean was used as the daily milk production of each group of cows during the pilot period; milk samples were drawn once a week for the determination of milk protein, milk fat, milk dry matter and somatic cell number. The statistical mean value was used as the basal milk composition parameter of the herd.

During the experimental period, milk production was measured once a day and the statistical mean value was used as the daily milk production of cows in each group during the experimental period. Milk samples were collected once a week for the determination of milk protein, milk fat, nonfat milk solid and the number of somatic cells. The statistical mean was used as the milk component value of each group of dairy cows during the experiment. The milk production was measured directly by the meter on the pipeline milking equipment and the milk components

Table 1: Major components of alfalfa extract.

Ingredient	Content (%)
Alfalfa polysaccharides	≥20
Alfalfa flavonoids	≥10
Alfalfa saponins	≥10
Starch	≥55
Water content	≥5

Table 2: Ingredients and nutrient composition of daily feed rations.

Diet composition (%)	Content (%)	Nutritional composition (%)	Content (%)
Whole plant corn silage	47.8	Net energy for milk production MJ/kg	7.94
Wet beer's grains	10.9	Crude protein	16.5
Alfalfa silage	10.9	Acid detergent fiber	18.3
Corn flour	12	long-chain fatty acids	3.1
Carex	6.5	Neutral detergent fiber	30.7
Flaked corn	2.2	Soluble fiber	7.2
Soybean meal	6.5	Lignin	2.9
DDGS	2	Silage acid	2.9
Baking soda	0.37	Sugar	3.5
Premix	0.35	Starch	29.9
Salt	0.2	Lys: Met	3.12:1
Stone powder	0.1		
Magnesium oxide	0.1		

were measured using an LMP2 P1 multifunctional automatic milk component analyzer and an electronic milk cell counter.

On Day 84 of the experiment, one cow was randomly selected from each replicate, with three cows sampled from each group. In total, 21 blood samples were collected across seven groups and blood was collected from the cows via the tail vein in the early morning before breakfast. The collected blood samples were centrifuged to obtain the serum, which was stored at -20°C until further examination. The level of IgG, IgA, IgM, IL-2, IL-6 and IL-10 in the serum of the cows in each group were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers′ instructions.

The experimental data were compiled *via* Excel 365 software and analyzed *via* one-way ANOVA using SPSSAU software. Duncan′s method was used for multiple comparisons and a histogram was drawn *via* GraphPad software. The results are expressed as the means±standard deviations and P<0.05 indicated a significant difference between groups.

RESULTS AND DISCUSION

Analysis of the differences in milk production and milk components among dairy cows in the pilot period

As shown in Table 3, the age, parity, milk production, physiological status, body condition and number of lactation days of the selected cows were basically the same before the experiment. During the 1-week pilot period, there was no significant difference in milk production between the groups of cows (P>0.05). As shown in Table 4, there were no significant differences in milk component content or somatic cell number among the various groups of cows during the preliminary test period (P>0.05).

Effects of alfalfa polysaccharides on milk production and milk composition in dairy cows

During the experimental period, the milk production of the dairy cows in the A0 group and the AP groups (AP1, AP2, AP3) were not significantly different (P>0.05). AE1, AE2 and AE3 cows had significantly greater milk production than did the A0 group and AP groups and the milk production of the AE2 group was significantly greater than that of the A0 group was (P<0.01). The number of somatic cells in the AE2 group was significantly lower than that in the A0 group (P<0.01) and the number of somatic cells in the AE group was significantly lower than that in the AP group (P>0.05). As shown in Fig 1 and Fig 2.

During the trial period, in the A0 group and the AP group, the milk fat percentages of the cows in each group were not significantly different (P>0.05). However, milk fat percentage in the AE2 group was significantly higher than that in the A0 group (P<0.05) and milk fat percentage in the AE1 and AE2 groups was higher than that in the A0 group, but the difference was not significant (P>0.05). The milk protein content of cows in the AP2 and AE2 groups was significantly greater than that in the AP0 group (P<0.05) and the milk protein content of the cows in the AP2 and AE groups tended to increase first and then decrease with increasing additive concentration. There was no significant difference in the nonfat milk solids percentage between the groups of cows (P>0.05) (Fig 3 and Table 5).

Table 3: Milk production of dairy cows in each group during the pilot period.

Groups	Quantity	Lactation (months)	Parity	Age	Milk production (kg)
A0	12	4	3	3.5	32.29±4.38
AP1	12	3	3.1	3.3	32.03±5.06
AP2	12	3	3.2	3.4	32.11±4.6
AP3	12	3	3	3.2	31.93±4.37
AE1	12	3	3.2	3.4	32.23±3.79
AE2	12	4	3.2	3.4	31.67±4.33
AE3	12	3	3.3	3.5	32.18±4.92

Table 4: Milk components of the cows in each group during the pilot period.

Groups	Milk fat (%)	Milk protein (%)	Nonfat milk solids (%)	Number of somatic cells($\times 10^3$/mL)	Acidity (°T)
AP0	4.48±0.21	2.77±0.16	8.35±0.58	121±21.3	16.5
AP1	4.38±0.34	2.76±0.21	8.54±0.63	124±29.2	16.3
AP2	4.41±0.19	2.63±0.15	8.41±0.52	131±33.18	16.7
AP3	4.33±0.28	2.54±0.2	8.47±0.61	136±34.26	16.4
AE1	4.53±0.35	2.64±0.24	8.57±0.78	127±31.3	16.3
AE2	4.41±0.33	2.66±0.15	8.55±0.72	129±24.83	16.2
AE3	4.34±0.27	2.81±0.27	8.73±0.67	125±27.44	16.0

The results are expressed as the means ± standard deviations.

A number of studies have shown that adding alfalfa or its extracts to livestock and poultry diets can effectively improve feed utilization efficiency, promote nutrient absorption and improve animal performance (Xia *et al*., 2021). Deng *et al*. (2023) reported that supplementation with alfalfa extract at 2 kg/t in the basal diet of beef cattle significantly increased the end weight and average daily weight gain of beef cattle, reduced the feed-to-weight ratio and promoted growth. Adding alfalfa to dairy cow rations can significantly increase the milk production of dairy cows and the milk fat percentage can be increased by at least 0.1% (Liu *et al*., 2019). Studies by Wang *et al*. (2016) have shown that alfalfa can improve the performance of pigs during the finishing stage of pig production. Zhan *et al*. (2018) reported that adding alfalfa flavones to the diet improved the feed intake of dairy cows and affected lactation performance by regulating hormone secretion. Our study revealed that the addition of various concentrations of alfalfa extract and polysaccharides (0, 1, 2, or 3 kg/t) to the basal diet of dairy cows effectively increased the milk protein content of dairy cows; however, there was a decreasing trend in the milk protein content after the concentration exceeded 2 kg/t. The increase in milk protein content caused by alfalfa extract was lower than that caused by alfalfa polysaccharide at the same concentration. We believe that these findings are related to the anti-inflammatory and antibacterial functions of alfalfa in improving immunity. The improvement effect of the alfalfa extract on the lactation performance of dairy cows was more comprehensive, as it increased the milk fat percentage and milk protein percentage, effectively increasing milk production and reducing the number of somatic cells. Therefore, alfalfa polysaccharides may not be the main substance in alfalfa that affects the lactation performance of dairy cows, but other active substances, such as alfalfa flavones and saponins, may effectively increase the milk protein content. Thus, this finding has reference value on how to further utilize alfalfa extract in cow feed.

Effects of alfalfa extract on the immune function of dairy cows

After 12 weeks of the experimental period, the immunity level of dairy cows in each group was detected. The results

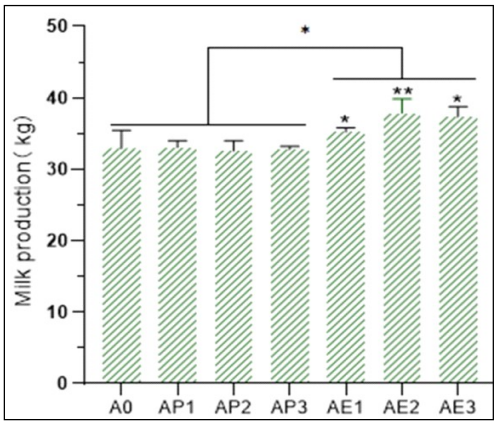


Fig 1: Determination of milk production in the experimental cows.

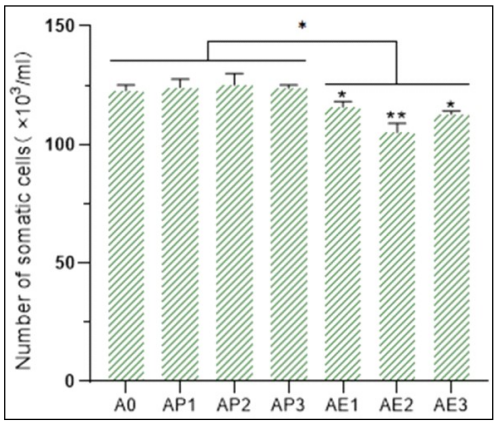


Fig 2: Determination of the number of somatic cells in the experimental cows.

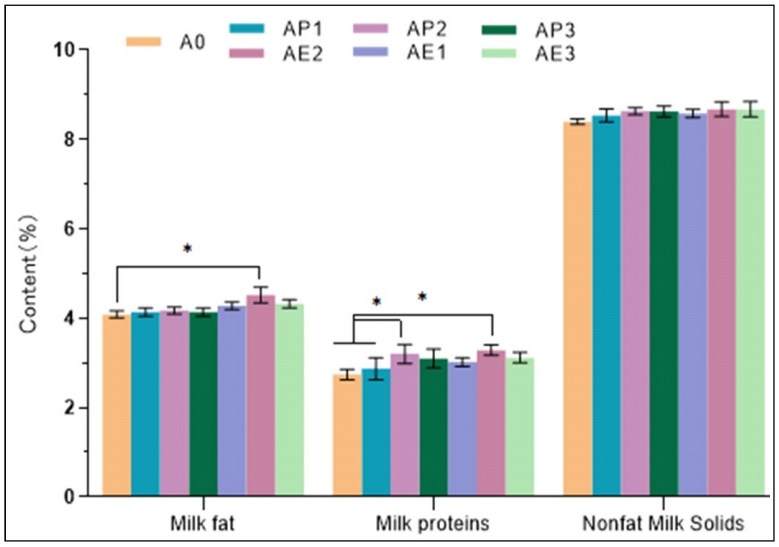


Fig 3: Determination of milk components in the experimental cows.

revealed that the IL-2 levels in the serum of dairy cows in the AP2, AP3, AE2 and AE3 groups were significantly greater than those of cows in the A0 and AP1 groups ($P<0.05$). The IL-2 levels in the serum of dairy cows in A0, AP1 and AE groups were similar ($P>0.05$). Additionally, the IgM antibody levels of dairy cows in the AP group and the AE group were significantly greater than those in the A0 group ($P<0.01$). The IL-10 levels in the serum of dairy cows in the A0, AP1 and AE1 groups were similar, while the IL-10 levels in the serum of the AP group and the AE group increased with increasing additive concentration and the difference was significant ($P <0.05$). Moreover, the IgA antibody level in the serum of dairy cows in the AP2, AP3, AE2 and AE3 groups was significantly greater than that in the A0, AP1 and AE1 groups ($P<0.01$). The IgG and IL-6 contents of dairy cows in the 7 groups were not significantly different ($P>0.05$). The levels of immune-related factors in the serum of dairy cows in the AP group were slightly greater than those in the serum of dairy cows in the AE group, but the difference was not significant ($P>0.05$) (Fig 4).

Alfalfa is rich in a variety of active substances that regulate immunity, such as alfalfa polysaccharides, flavones and saponins. Studies have shown that alfalfa polysaccharides have certain regulatory effects on the immune function of animals and have anti-inflammatory and antibacterial effectsg (Li *et al*., 2020; Gözüaçık *et al*., 2017). Alfalfa polysaccharides can bind to the mouse TLR4 membrane receptor to activate the MAPK and NF-κB pathways, inducing the expression and release of cytokines and various inflammatory factors, such as IL-1 and COX-2, promoting the proliferation of T, B and natural killer (NK) cells and improving immunity (Boivin *et al*., 2013). Xin *et al*. (2016) reported that alfalfa polysaccharides exert immune effects mainly by regulating serum IL-10 and NO secretion and iNOS activity. Moreover, Zhang *et al*. (2014) reported that an appropriate amount of alfalfa polysaccharide could significantly enhance the immune effect of attenuated Newcastle disease vaccines. Zhao *et al*. (2005) reported that alfalfa polysaccharides can be used as enhancers of classical swine fever rabbit vaccines and can increase the number of B lymphocytes and serum antibody levels in piglets. Our experimental results revealed that adding alfalfa extract and polysaccharides (1, 2, or 3 kg/t) at graded concentrations to the basal diet of dairy cows significantly increased the IgM content in dairy cow serum and that high concentrations had a significant effect on increasing IgA level in dairy cow serum. These results indicated that a certain level of alfalfa extract or polysaccharide could

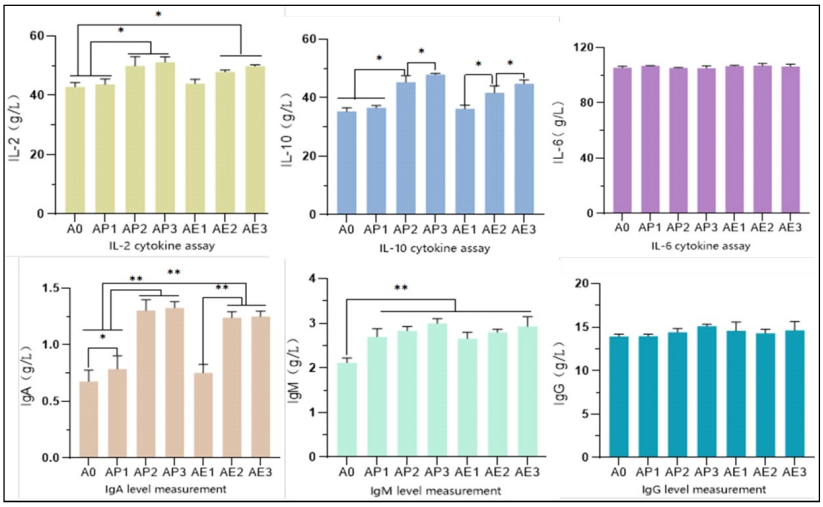


Fig 4: Determination of immune factor levels in experimental dairy cows.

Table 5: Milk components of the cows in each group during the experimental period.

Groups	Milk fat (%)	Milk protein (%)	Nonfat milk solids (%)	Number of somatic cells(×10³/mL)	Acidity (°T)	Milk production (kg)
AP0	4.08±0.22	2.74±0.19	8.4±0.12	123±4.8	16.3	32.89±3.46
AP1	4.13±0.21	2.87±0.28	8.54±0.31	124±6.46	16.2	33.05±1.27
AP2	4.17±0.18	3.2±0.23	8.63±0.14	125±8.36	16.3	32.56±3.21
AP3	4.14±0.2	3.1±0.25	8.63±0.21	124±2.47	16.4	32.75±2.77
AE1	4.28±0.14	3.02±0.26	8.58±0.24	116±3.19	15.8	35.23±1.04
AE2	4.52±0.29	3.29±0.17	8.68±0.4	105±5.22	16.1	37.84±3.2
AE3	4.33±0.2	3.12±0.15	8.69±0.38	113±2.86	16.2	37.34±2.51

The results are expressed as the means ± standard deviations.

effectively improve the immune performance of dairy cows. Although a significant difference was not detected, the improvement in the immune performance of dairy cows caused by the alfalfa polysaccharides was greater than that caused by the alfalfa extract at the same concentration. Therefore, we believe that alfalfa polysaccharides might be the main substances in alfalfa that affect the immune function of dairy cows.

Cytokines play important roles in mediating the immune response and can be involved in maintaining tissue integrity. Th1 cells can secrete cytokines such as IL-2, IFN- γ and TNF- β; Th2 cells can secrete cytokines such as IL-4, IL-5, IL-6 and IL-10; and Th0 cells can produce Th1 cytokines as well as Th2 cytokines (Ridhowi *et al*., 2017). Plant polysaccharides can regulate the body's immune function by affecting the secretion levels of various cytokines. The results of this study revealed that alfalfa extract and polysaccharides could significantly increase the levels of IL-2 and IL-10 in dairy cow serum, while IL-2 can activate T cells and promote the proliferation of B cells and the secretion of antibodies. Additionally, IL-10 can inhibit the inflammatory response and increase B-cell survival. Therefore, alfalfa extract and polysaccharides can significantly improve the immunity of dairy cows and the mechanism of action may involve the regulation of cellular immunity. These findings lay the foundation for further studies on the regulatory mechanisms of alfalfa in dairy cow immunity.

Conclusion

Under the conditions of this study, alfalfa extract improved both the lactation performance and immunity of dairy cows, indicating suitable comprehensive effects. Alfalfa polysaccharides appeared to affect the immunity of dairy cows more than the lactation performance. On the basis of the effects and economic value of alfalfa extract, we recommend its use at the optimal concentration of 2 kg/t in dairy farming.

Acknowledgement

The present study was supported by Scientific Research Project of Hunan Provincial Department of Education. Project No. 22C0734

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest statement

The all authors of the manuscript declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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