

In vitro effects of alpha-ketoglutarate and folic acid supplementation on bull sperm exposed to ammonia stress

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Abstract

This study examined the effects of alpha-ketoglutaric acid (AKG; 2.00, 4.00, and 8.00 mM L⁻¹) and folic acid (FA; 50.00 nM L⁻¹) on bull sperm cells under ammonia stress induced by 175 μM L⁻¹ ammonium chloride. Sperm parameters including kinematic motility, survival rate, membrane integrity, DNA integrity, superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), total antioxidant capacity (TAC) activities, and apoptosis were assessed in the groups. Sperm motility indices, survival rate, plasma membrane integrity, SOD, CAT, TAC, and GPx enzymes activity, DNA damage, and apoptosis in the treated groups were significantly differed from those in the control groups. The AKG concentrations of 4.00 and 8.00 mM L⁻¹ co-supplemented with 50.00 nM L⁻¹ FA mitigated the negative effects of ammonia on sperm cells. This study indicated that supplementation with AKG and FA at the desired concentrations counteracted the adverse effects of ammonia toxicity that preceded clinical signs. Further studies are needed to evaluate the fertility of these sperms, either *in vitro* or *in vivo*.

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Introduction

Toxic metabolites of non-protein- nitrogen pose an important danger and a notable threat to animal health. Ruminant microflora produces amino acids and microbial proteins using keto acids from carbohydrate fermentation and ammonia from nonprotein nitrogen sources. Research reports indicated that high urea concentrations reduces conception rates and fertility of the gametes and embryos.¹ Ammonia and ammonium maintain an equilibrium within the body. However, when the ammonia intake surges, the excess amount absorbs from the digestive tract into the bloodstream. Clinical signs of ammonia toxicity are nonspecific and its severity often poorly correlates with the degree of hyperammonemia. Therefore, the diagnosis of subacute ammonia toxicity is clinically insignificant.² In a healthy liver, nearly all intestinal ammonia entering the bloodstream is removed during the first-round metabolism. Ammonia absorption from the rumen into the bloodstream escalates with higher rumen concentration or pH.³ Various reports have indicated that excessive nitrogen concentrations can adversely affect reproduction,^{4,5} while another study showed that a wide range of urea

concentrations is associated with good fertility.⁶ However, the lack of clinical signs and high body tolerance does not guarantee the absence of subclinical damage to germ cells. Folic acid (FA) improves semen quality and spermatogenesis by influencing autophagy and histone methylation in aged broilers.⁷ It protects chromatin quality, membrane integrity, and sperm viability. The FA also helps maintain sperm parameters after freezing,⁸ and positively affects the DNA fragmentation index when combined with other micronutrients.⁹ Thus, its potential to mitigate the effects of subclinical ammonia concentrations on sperm cells is worth exploring.

Alpha-ketoglutaric (AKG), a key molecule in the citric acid cycle, plays some roles in oxidation reactions and energy production.¹⁰ It also contributes to glutamine and glutamate synthesis, supports protein synthesis, prevents protein degradation in muscle, and acts as a metabolic fuel for digestive tract cells.¹¹ As an antioxidant and energy source, AKG capacity to increase antioxidant levels by strengthening glutamine synthesis is noteworthy.¹² Bearing in mind that ammonium and ammonia are in chemical balance in aqueous solutions, the ammonia effects can be evaluated *in vitro*.⁵ Studies have shown the

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toxic effects of ammonia in humans and animals expressing clinical signs. Toxic effects of ammonium chloride (AC) on human sperm at a concentration of 914 $\mu\text{M L}^{-1}$ have been shown.¹³ The incidence of a dramatic decrease in mobility and live sperm count of bulls lacking clinical signs and those with ammonia blood concentrations much lower than the reported values in a commercial stud breeding center provoked the idea whether the lower concentration of ammonia could affect sperm parameters. The aim of this study was to evaluate the potential of alpha-ketoglutarate and FA in ameliorating the toxicity associated with elevated ammonia concentrations on bull sperm cool preservation *in vitro*.

Materials and Methods

Study location and semen processing .This study was carried out in Tabriz genetic material production center (38° 01'42''N 46°06'53''E, altitude 1390m) with dry steppe climate. The amount of rainfall is 513 to 192 mm *per* year with the humidity is 28.00 - 44.00 %. The maximum and minimum temperature is 25.40 and - 2.50 °C, respectively. The study complied with the Animal Research: Reporting of *In Vivo* Experiments guidelines and was approved by the Iran National Committee for Ethics in Biomedical Research (IR.TABRIZU.REC.1402.087). The collection of semen samples was routinely performed twice a week from five Simmental bulls (aged 4 - 6 years) using an artificial vagina and mixed together. To avoid the individual differences all samples from the bulls were pooled and the volume, color, possible contamination, concentration and progressive motility were assessed. The samples having characteristics of 85.00% motility, four grade progressive motility, over 2.50×10^9 *per* mL sperm count and reasonable consistency were chosen for the experiment. The pH and osmolality were controlled in all treatment groups. The samples were diluted in Tris-glycerol-egg extender [Tris (hydroxymethyl) amino methane 250 mM L⁻¹, citric acid 90.00 mM L⁻¹, fructose 70.00 mM L⁻¹, glycerol 7.00% (v/v), egg yolk 20.00% (v/v), streptomycin 100 $\mu\text{g mL}^{-1}$, and penicillin G Procaine 100 IU mL⁻¹].¹⁴ In addition, 10.00 mL of blood was collected from the bulls via the coccygeal vein using a Venoject® tube containing anticoagulant (dipotassium ethylenediamine-tetraacetic acid). The blood samples were centrifuged at 5,000 rpm for 10 min. The plasma samples were collected in less than 30 min and frozen in liquid nitrogen until analysis. All chemicals were obtained from Sigma-Aldrich (St. Louis, USA) unless otherwise stated.

Threshold of ammonia toxicity limit. After equilibration at 2.00 - 8.00 °C for 2 hr in Tris-glycerol-egg extender, the sperm cells were exposed to 50.00, 100, 150, 175, 200, 250, 300, 350 $\mu\text{M L}^{-1}$ AC and examined 0, 5, 15, and 30 min later. The solvent for AC was distilled water. The sperm kinematic motilities were evaluated using

Computer-Assisted Sperm Analysis (HFT Sperm Analyzer V741; Hooshmand Fanavar Tehran Co., Tehran, Iran). According to the results (data are not presented) the effective concentration of ammonia stress was 175 $\mu\text{M L}^{-1}$ at 30 min of addition.

Chemical preparation and experimental design.

The FA was dissolved in dimethyl sulfoxide and used at a concentration of 50.00 nM L⁻¹.¹⁵ The AKG was dissolved in distilled water, and concentrations of 2.00, 4.00, and 8.00 mM L⁻¹ were prepared.¹⁶ The AC was dissolved in water and prepared at a concentration of 175 $\mu\text{M L}^{-1}$. The AKG, FA and AC were added into nine experimental groups at a final concentration of 40.00×10^6 spermatozoa *per* mL as follows: 1 (control 1) = AKG 0.00 mM L⁻¹ + FA 0.00 nM L⁻¹ + AC 0.00 $\mu\text{M L}^{-1}$; 2 (control 2) = AKG 0.00 mM L⁻¹ + FA 0.00 nM L⁻¹ + AC 175 $\mu\text{M L}^{-1}$; 3 = AKG 2.00 mM L⁻¹ + FA 0.00 nM L⁻¹ + AC 175 $\mu\text{M L}^{-1}$; 4 = AKG 4.00 mM L⁻¹ + FA 0.00 nM L⁻¹ + AC 175 $\mu\text{M L}^{-1}$; 5 = AKG 8.00 mM L⁻¹ + FA 0.00 nM L⁻¹ + AC 175 $\mu\text{M L}^{-1}$; 6 = AKG 0.00 mM L⁻¹ + FA 50.00 nM L⁻¹ + AC 175 $\mu\text{M L}^{-1}$; 7 = AKG 2.00 mM L⁻¹ + FA 50.00 nM L⁻¹ + AC 175 $\mu\text{M L}^{-1}$; 8 = AKG 4.00 mM L⁻¹ + FA 50.00 nM L⁻¹ + AC 175 $\mu\text{M L}^{-1}$; 9 = AKG 8.00 mM L⁻¹ + FA 50.00 nM L⁻¹ + AC 175 $\mu\text{M L}^{-1}$. At least three replicates were performed for all experimental groups.

Blood ammonia assay. Ammonia concentrations were measured by spectrophotometry (Hitachi, Tokyo, Japan) using a commercial kit (Randox Teoranta, Dungloe, Ireland). The optical absorption of the sample at 340 nm wavelength was compared with the standard sample to determine the amount of ammonia.¹⁷

Assessment of sperm motility. Sperm kinematic parameters, including total motility (TM%), progressive motility (PM%), speed along a straight-line path (VSL; $\mu\text{m per sec}$), speed along a curved path (VCL; $\mu\text{m per sec}$), average along-path velocity (VAP; $\mu\text{m per sec}$), Amplitude of lateral head displacement (ALH; μm), average angular displacement (MAD; degree), linearity (%), wobble (%), straightness (%) and beating cross frequency (Hz),¹⁸ were analyzed using Computer-Aided Sperm Analysis. A volume of 10.00 μL of each sample was placed on a slide heated to 35.00 °C (Leja, Nieuw-Vennep, Netherlands). Each slide, containing a minimum of 200 sperm cells, was evaluated using a camera (Samsung, Seoul, Korea) capturing images at a rate of 50 *per* sec in five fields, with 10 × magnification provided by a phase-contrast microscope (PROWAY, Shanghai, China).

Evaluation of survival rate. The viability of sperm cells was assessed using Eosin-Nigrosine staining (Eosin 16.70 g L⁻¹, Nigrosine 100 g L⁻¹, and trisodium citrate 29.20 g L⁻¹). Ten microliters of sperm were mixed with the same volume of dye solution on the slide. Afterward, a smear was prepared, and the slides were dried at room temperature. At least 200 sperm cells in each sample were evaluated using an inverted phase contrast microscope (Labomed, Los Angeles, USA).¹⁹

Evaluation of sperm plasma membrane integrity.

Thirty microliters of sperm with 300 μL of a hypo-osmotic solution (13.50 g L^{-1} fructose and 7.35 g L^{-1} sodium citrate, 100 mOsm L^{-1}) were incubated at 35.00 $^{\circ}\text{C}$ for 60 min. Then, at least 200 spermatozoa were evaluated at a magnification of 400 \times using a phase-contrast microscope in at least three microscopic fields on each slide.²⁰ Spermatozoa with intact membranes swelled within 5 minutes in the hypo-osmotic solution, and all flagellar shapes stabilized within 30 min.²¹

Evaluation of DNA damage. The DNA integrity evaluation was performed using Acridine Orange staining. Briefly, 100 μL of the sample was centrifuged and washed twice with 500 μL of phosphate-buffered saline (Na_2HPO_4 : 1.4196 g, KH_2PO_4 : 0.2449 g, KCl : 0.2013 g, NaCl : 8.0067 g, in 1.00 L distilled water, pH 7.20) at a speed of 1000 rpm for 5 min. Then, the pellet was resuspended in 50.00 μL of phosphate-buffered saline, and a 20.00 μL smear was prepared and dried at 35.00 $^{\circ}\text{C}$. All smears were fixed by immersion in Carnoy's fluid fixation (three volumes methanol and one volume of glacial acetic acid) at 2.00 to 8.00 $^{\circ}\text{C}$ for at least 2 hr.²² After fixation, the smears were washed with distilled water, placed in Acridine Orange dye solution for 7 to 10 min at room temperature, and shielded from light. The evaluation of DNA damage was carried out using an inverted fluorescence microscope (Nikon, Tokyo, Japan).

Flow cytometry analysis and assessment of cell apoptosis. Cell apoptosis was assessed through flow cytometry using the ApoflowEx-fluorescein isothiocyanate (FITC) kit ApoFlowEx FITC Kit (Exbio Praha, Vestec, Czech Republic) and the Beckman Coulter Life Sciences device (Beckman Coulter Life Sciences, Indianapolis, USA). The kit included Annexin V binding buffer with a concentration 10 times higher than that of the original components (0.10 M HEPES/NaOH, pH 7.40, 1.40 M NaCl, 25.00 mM CaCl_2), two-color reagents, Annexin V-FITC, and propidium iodide (PI) solution. The flow cytometry device employed blue laser light with an excitation wavelength of 488 nm and emitted or re-emitted light at 525 nm for FITC and 617 nm for PI. Flow cytometry graphs were generated using FlowJo v10 (Becton Dickinson and Company, Franklin Lakes, USA). The X-axis represents fluorescence intensity in the FITC channel (FL1), and the Y-axis represents the PI channel (FL3). In each experiment, more than 10,000 cellular events were analyzed, categorizing them as viable, early apoptotic, late apoptotic, and dead cell populations. The cell gate for analysis was set to evaluate a minimum of 5,000 to 10,000 cells. To establish the gate, the negative control sample was checked at the beginning of the analysis. All measurements were performed in triplicates. Apoptotic cells exhibited high fluorescence intensity in the FITC channel, while necrotic (dead) and late apoptotic cells displayed high fluorescence intensity in both the FITC and PI channels.²³

Oxidation of spermatozoa analysis. Enzyme tests were performed using commercial kits (Navand Salamat, Urmia, Iran). Superoxide dismutase (SOD), Glutathione peroxidase (GPx) and total antioxidant capacity (TAC),²⁴ activities were measured at a wavelength of 405, 340 and 630 nm, respectively, using commercial kits (Navand Salamat) and a microreader enzyme-linked immunosorbent assay device (Dana 3200; Garni Medical Engineering Co., Tehran, Iran). The enzyme activity of catalase (CAT) was measured at a wavelength of 550 nm using an enzyme-linked immunosorbent assay microreader (MPR4++; Hyperion, Roedermark, Germany).²⁵

Statistical analysis. All data were statistically analyzed using SPSS Software (version 23.0; IBM Corp., Armonk, USA) and the results are reported as the mean \pm standard error. The effects of treatments were analyzed using generalized linear model. The supplement factors were coded with orthogonal contrasts to guarantee that each independent variable delineated a distinct portion of the variation in the results. For comparisons among the treatments, three independent contrasts were required. Contrast 1 (including three individual models) compared the effect of all substances (AM, AKG and FA) with the control group. In contrast 2 (including three pairs models), the interaction effects of the supplements were compared in pairs (AKG \times FA), (AM \times FA) and (AM \times AKG). Finally, contrast three compares the simultaneous interaction of all three substances (AM \times AKG \times FA). Differences were considered significant when the p value was less than 0.05.

Results

Effects of different concentrations of ammonium chloride on sperm cells and blood ammonia. The results (data are not presented) indicated that different concentrations of AC at various time points affect sperm kinematic parameters. However, significant values were observed after 30 min of incubation at 175 $\mu\text{M L}^{-1}$. The average of bulls' blood ammonia concentration was 60.00 $\mu\text{M L}^{-1}$. Ammonium chloride at this concentration causes an increase in immotile sperm and a significant decrease in the movement speed of motile sperm cells.

Interaction effects. The results of the interaction of supplemented agents to Simmental bull sperm (Table 1) showed that the significant difference in sperm kinetic parameters under AC stress was mainly due to the effect of AKG rather than FA. In contrast, the significant difference in antioxidant enzymes activity and TAC was more influenced by FA than AKG. Additionally, the results of statistical models indicated a significant difference in parameters of cell survival rate, plasma membrane integrity, apoptosis, and DNA damage related to the interaction of AKG and FA.

Table 1. The *p* values for the main and interaction effects of ammonium chloride (AC; 0.00 and 175 $\mu\text{M L}^{-1}$), alpha-ketoglutarate (AKG; 0.00, 2.00, 4.00 and 8.00 mM L^{-1}) and folic acid (FA; 0.00 and 50.00 nM L^{-1}) supplemented to the chilled Simmental bull sperm.

Parameters	Contrast 1			Contrast 2			Contrast 3
	AC	AKG	FA	AKG \times FA	AC \times AKG	AC \times FA	AC \times AKG \times FA
TM	0.00	0.00	0.19	0.00	0.00	0.00	0.00
PM	0.00	0.00	0.08	0.00	0.01	0.96	0.00
IM	0.00	0.00	0.29	0.00	0.00	0.00	0.00
VCL	0.00	0.00	0.70	0.00	0.00	0.04	0.00
VAP	0.00	0.00	0.42	0.00	0.00	0.45	0.00
VSL	0.00	0.00	0.59	0.00	0.02	0.42	0.01
BCF	0.00	0.00	0.09	0.00	0.00	0.03	0.00
MAD	0.00	0.00	0.67	0.00	0.00	0.47	0.00
ALH	0.00	0.00	0.07	0.00	0.00	0.00	0.00
LIN	0.02	0.00	0.15	0.00	0.43	0.00	0.05
WOB	0.08	0.03	0.43	0.57	0.17	0.92	0.50
SRT	0.02	0.00	0.15	0.00	0.62	0.00	0.00
SOD	0.00	0.62	0.00	0.99	0.89	0.00	0.89
CAT	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TAC	0.00	0.21	0.01	0.06	0.18	0.19	0.50
GPX	0.04	0.17	0.03	0.07	0.06	0.00	0.04
Q1	0.00	0.00	0.00	0.00	0.00	0.71	0.00
Q2	0.21	0.00	0.00	0.00	0.00	0.26	0.00
Q3	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Q4	0.00	0.07	0.03	0.02	0.00	0.00	0.00
SR	0.00	0.10	0.90	0.04	0.00	0.07	0.11
HOST	0.14	0.06	0.65	0.00	0.00	0.10	0.01
DNA damage	0.00	0.00	0.00	0.01	0.00	0.00	0.02

TM: Total motility, PM: Progressive motility, IM: Immotile, VCL: Velocity of curved line, VAP: Velocity of average path, VSL: Velocity of straight line, BCF: Beat cross frequency, MAD: Mean angle degree, ALH: Amplitude of lateral head displacement, SOD: Superoxide dismutase, CAT: Catalase, TAC: Total antioxidant capacity, GPx: Glutathione peroxidase, Q1: Necrotic cells, Q2: Late apoptotic cells, Q3: Early apoptotic cells, Q4: Non-apoptotic cells, SR: Survival rate, and HOST: Hypo-osmotic swelling test.

Sperm motility. The sperm motion kinematic results are presented in Table 2. The mean \pm SEM values for TM, PM, VCL, VAP, VSL, and ALH in all experimental groups were increased compared to the control group. While the mean \pm SEM for IM decreased ($p < 0.05$) in all groups compared to the relevant control groups 1 and 2. The beating cross frequency results showed that the experimental groups 3 - 6 and 8 had increased values ($p < 0.05$) compared to the control group 2 (1.97 ± 0.07). Moreover, the mean \pm SEM for MAD in all experimental groups, except groups 4 (19.32 ± 0.95) had increased values ($p < 0.05$) compared to the control 2 (16.63 ± 0.79). The mean \pm SEM linearity was higher ($p < 0.05$) in groups 3 (30.36 ± 0.61), 4 (29.21 ± 0.93), 5 (32.67 ± 1.15) and 8 (28.81 ± 0.87) than in the control groups 1 (25.04 ± 0.6) and 2 (25.45 ± 0.77). The wobble result showed that the experimental group 4 (51.89 ± 0.9) had increased value ($p < 0.05$) compared to control group 2 (45.33 ± 0.105). The straightness was also higher ($p < 0.05$) in groups 3 (57.07 ± 1.56) and 5 (55.74 ± 1.23) than the control groups 1 (51.8 ± 0.53) and 2 (51.92 ± 0.84). While, the groups 7 (50.00 ± 0.85) and 9 (50.32 ± 0.84) had decreased straightness values ($p < 0.05$) compared to the control groups 1 and 2.

Enzyme activity. The results for enzyme activities of SOD, CAT, TAC and GPx are shown in Figure 1. The mean \pm

SEM of the SOD enzyme activity decreased ($p < 0.05$) in groups 6 (194.51 ± 2.53), 7 (188.47 ± 1.76), 8 (191.59 ± 3.51) and 9 (189.62 ± 5.56) compared to the control groups 1 (216.56 ± 08.57). The groups 6 (0.52 ± 0.00) and 9 (0.54 ± 0.00) had increased values ($p < 0.05$) of CAT compared to the control groups 1 (0.52 ± 0.00) and 2 (0.51 ± 0.00). The TAC activity increased ($p < 0.05$) in all groups, except groups 3 (933.33 ± 140.35) and 4 (807.08 ± 182.94) compared to the control groups 1 (771.72 ± 83.69) and 2 (897.98 ± 32.89). The GPx significantly ($p < 0.05$) lowered in groups 1 (300.55 ± 12.75) compared to all other groups.

Apoptosis. The results of the flow cytometry assay are shown in Table 3 and Figure 2. The number of live cells was significantly ($p < 0.05$) increased in the groups 3 - 9 compared to the control group 2 (84.08 ± 0.48). In contrast, the groups 5 - 8 had decreased ($p < 0.05$) early apoptotic cells ($p < 0.05$) compared to control group 2 (08.88 ± 0.39). Furthermore, the mean \pm SEM of late apoptotic cells in groups 4 (1.91 ± 0.05), 5 (01.92 ± 00.05), 6 (1.40 ± 0.07), 8 (1.64 ± 0.23) and 9 (1.46 ± 0.18) were decreased ($p < 0.05$) compared to their relative control group 2 (2.35 ± 0.12). All experimental groups expect 7 (4.38 ± 0.68) had significantly ($p < 0.05$) fewer necrotic cells than the control group 2 (4.69 ± 0.25).

Table 2. Mean ± SEM values of kinematic motility parameters of the Simmental bull chilled-spermatozoa treated with ammonium chloride (AC; 0.00 and 175 µM L⁻¹), alpha-ketoglutarate (AKG; 0.00, 2.00, 4.00 and 8.00 mM L⁻¹) and folic acid (FA; 0.00 and 50.00 nM L⁻¹).

Parameters	1	2	3	4	5	6	7	8	9
TM	56.73 ± 1.04d	40.82 ± 1.77a	75.18 ± 2.10b	57.68 ± 1.78c	58.52 ± 1.42c	54.46 ± 0.85d	57.08 ± 0.80cd	69.22 ± 1.69e	55.34 ± 1.14cd
PM	46.08 ± 1.08df	33.80 ± 1.39a	64.16 ± 2.70b	47.55 ± 2.10cd	49.94 ± 1.83c	44.34 ± 0.96df	44.87 ± 0.90df	57.81 ± 1.49e	43.86 ± 1.32f
IM	43.27 ± 1.04cd	59.18 ± 1.77a	24.82 ± 2.10b	42.61 ± 1.79cd	41.93 ± 1.39c	45.54 ± 0.85d	42.95 ± 0.78cd	29.79 ± 1.21e	44.70 ± 1.14cd
VCL	35.55 ± 0.55e	25.97 ± 0.88a	43.16 ± 1.33b	30.94 ± 0.79c	33.04 ± 0.96de	32.86 ± 1.29e	33.69 ± 0.95de	38.81 ± 2.04d	33.29 ± 1.57de
VAP	16.38 ± 0.63c	11.96 ± 0.55a	21.73 ± 0.90b	15.70 ± 0.47c	17.59 ± 0.85c	16.13 ± 0.78c	16.38 ± 0.55c	19.70 ± 1.20d	16.96 ± 0.86c
VSL	10.20 ± 0.55c	7.61 ± 0.40a	14.51 ± 0.75b	10.29 ± 0.45c	12.56 ± 0.92de	10.55 ± 0.73c	10.11 ± 0.56c	12.92 ± 1.14bd	11.03 ± 0.67ce
BCF	1.97 ± 0.07c	1.34 ± 0.08a	3.04 ± 0.21b	1.83 ± 0.12c	1.97 ± 0.15c	1.76 ± 0.16c	1.73 ± 0.15ac	2.04 ± 0.16c	1.56 ± 0.17a
MAD	25.01 ± 0.37d	16.63 ± 0.79a	31.72 ± 1.16b	19.33 ± 0.96ac	20.76 ± 0.96c	21.58 ± 1.31c	21.82 ± 1.30c	25.54 ± 1.72d	20.16 ± 1.34c
ALH	2.43 ± 0.02f	1.96 ± 0.06d	2.64 ± 0.04b	2.18 ± 0.02c	2.20 ± 0.03c	2.22 ± 0.03c	2.36 ± 0.02d	2.56 ± 0.06e	2.33 ± 0.05d
STR	51.80 ± 0.53ac	51.92 ± 0.84ac	57.07 ± 1.56b	53.13 ± 0.67a	55.74 ± 1.23b	52.60 ± 0.70a	50.00 ± 0.85c	54.12 ± 1.35ab	50.32 ± 0.84c
LIN	25.04 ± 0.60a	25.45 ± 0.77a	30.36 ± 1.61b	29.21 ± 0.93b	32.67 ± 1.15c	26.96 ± 0.49a	26.13 ± 0.63a	28.81 ± 0.87bd	26.96 ± 0.63ad
WOB	46.22 ± 0.84abc	45.33 ± 1.05ac	50.81 ± 1.16ab	51.89 ± 0.90b	44.26 ± 8.55c	48.50 ± 0.34abc	48.72 ± 0.24abc	49.97 ± 0.55abc	48.74 ± 0.74abc

TM: Total motility, PM: Progressive motility, IM: Immotile, VCL: Velocity of curved line, VAP: Velocity of average path, VSL: Velocity of straight line, BCF: Beat cross frequency, MAD: Mean angle degree, ALH: Amplitude of lateral head displacement, STR: Straightness, LIN: Linearity, and WOB: Wobble.

Numbers in the first column refer to the groups as follows: 1 (control 1) = AKG 0.00 mM L⁻¹ + FA 0.00 mM L⁻¹ + AC 0.00 µM L⁻¹; 2 (control 2) = AKG 0.00 mM L⁻¹ + FA 0.00 mM L⁻¹ + AC 0.00 mM L⁻¹ + AC 175 µM L⁻¹; 3 = AKG 2.00 mM L⁻¹ + FA 0.00 mM L⁻¹ + AC 175 µM L⁻¹; 4 = AKG 4.00 mM L⁻¹ + FA 0.00 mM L⁻¹ + AC 175 µM L⁻¹; 5 = AKG 8.00 mM L⁻¹ + FA 0.00 mM L⁻¹ + AC 175 µM L⁻¹; 6 = AKG 0.00 mM L⁻¹ + FA 50.00 nM L⁻¹ + AC 175 µM L⁻¹; 7 = AKG 2.00 mM L⁻¹ + FA 50.00 nM L⁻¹ + AC 175 µM L⁻¹; 8 = AKG 4.00 mM L⁻¹ + FA 50.00 nM L⁻¹ + AC 175 µM L⁻¹; 9 = AKG 8.00 mM L⁻¹ + FA 50.00 nM L⁻¹ + AC 175 µM L⁻¹.

^{a-d}Values within a column with different superscript letters indicate statistically differences (*p* < 0.05).

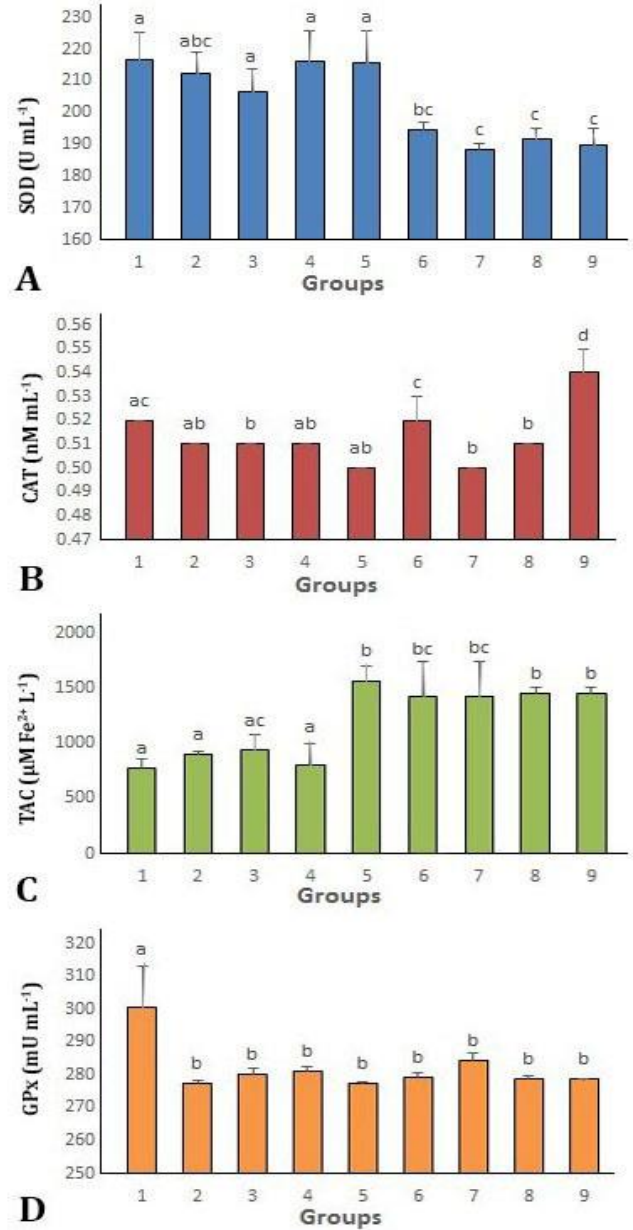


Fig. 1. Mean ± SEM values of **A)** Superoxide dismutase (SOD), **B)** Catalase (CAT), **C)** Total antioxidant capacity (TAC), and **D)** Glutathione peroxidase (GPx), enzyme activities of Simmental bull chilled spermatozoa treated with ammonium chloride (AC; 0.00 and 175 µM L⁻¹), alpha-ketoglutarate (AKG; 0.00, 2.00, 4.00 and 8.00 mM L⁻¹) and folic acid (FA; 0.00 and 50.00 nM L⁻¹). Numbers refer to groups as 1 (control 1) = AKG 0.00 mM L⁻¹ + FA 0.00 nM L⁻¹ + AC 0.00 µM L⁻¹; 2 (control 2) = AKG 0.00 mM L⁻¹ + FA 0.00 nM L⁻¹ + AC 175 µM L⁻¹; 3 = AKG 2.00 mM L⁻¹ + FA 0.00 nM L⁻¹ + AC 175 µM L⁻¹; 4 = AKG 4.00 mM L⁻¹ + FA 0.00 nM L⁻¹ + AC 175 µM L⁻¹; 5 = AKG 8.00 mM L⁻¹ + FA 0.00 nM L⁻¹ + AC 175 µM L⁻¹; 6 = AKG 0.00 mM L⁻¹ + FA 50.00 nM L⁻¹ + AC 175 µM L⁻¹; 7 = AKG 2.00 mM L⁻¹ + FA 50.00 nM L⁻¹ + AC 175 µM L⁻¹; 8 = AKG 4.00 mM L⁻¹ + FA 50.00 nM L⁻¹ + AC 175 µM L⁻¹; 9 = AKG 8.00 mM L⁻¹ + FA 50.00 nM L⁻¹ + AC 175 µM L⁻¹. ^{a-d}Values within a column with different superscript letters differ significantly (*p* < 0.05).

Table 3. Mean \pm SEM values of cell apoptosis of Simmental bull chilled spermatozoa treated with ammonium chloride (AC; 0.00 and 175 $\mu\text{M L}^{-1}$), alpha-ketoglutarate (AKG; 0.00, 2.00, 4.00, and 8.00 mM L^{-1}) and folic acid (FA; 0.00 and 50.00 nM L^{-1}).

Groups	Q1	Q2	Q3	Q4
1	2.58 \pm 0.18 ^{ac}	2.47 \pm 0.17 ^a	5.36 \pm 0.17 ^f	89.59 \pm 0.46 ^f
2	4.69 \pm 0.25 ^{be}	2.35 \pm 0.12 ^a	8.88 \pm 0.39 ^a	84.08 \pm 0.48 ^a
3	1.96 \pm 0.15 ^{ad}	2.18 \pm 0.08 ^a	8.39 \pm 0.12 ^{abe}	87.47 \pm 0.31 ^{be}
4	2.70 \pm 0.16 ^c	1.91 \pm 0.05 ^b	8.62 \pm 0.15 ^{ab}	86.77 \pm 0.17 ^b
5	1.81 \pm 0.15 ^d	1.92 \pm 0.05 ^b	8.19 \pm 0.18 ^b	88.08 \pm 0.22 ^{bc}
6	3.29 \pm 0.16 ^{cf}	1.40 \pm 0.07 ^c	6.38 \pm 0.15 ^{ce}	88.92 \pm 0.11 ^{cf}
7	4.38 \pm 0.68 ^{ef}	2.38 \pm 0.34 ^a	7.93 \pm 0.24 ^d	85.31 \pm 0.85 ^d
8	3.79 \pm 0.55 ^f	1.64 \pm 0.23 ^{bc}	5.957 \pm 0.17 ^c	88.62 \pm 0.84 ^{cef}
9	3.27 \pm 0.39 ^{cf}	1.46 \pm 0.18 ^c	6.84 \pm 0.11 ^e	88.43 \pm 0.67 ^{cef}

Group 1 (control 1) = AKG 0.00 mM L^{-1} + FA 0.00 nM L^{-1} + AC 0.00 $\mu\text{M L}^{-1}$; Group 2 (control 2) = AKG 0.00 mM L^{-1} + FA 0.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$; Group 3 = AKG 2.00 mM L^{-1} + FA 0.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$; Group 4 = AKG 4.00 mM L^{-1} + FA 0.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$; Group 5 = AKG 8.00 mM L^{-1} + FA 0.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$; Group 6 = AKG 0.00 mM L^{-1} + FA 50.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$; Group 7 = AKG 2.00 mM L^{-1} + FA 50.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$; Group 8 = AKG 4.00 mM L^{-1} + FA 50.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$; Group 9 = AKG 8.00 mM L^{-1} + FA 50.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$. Q1 = Necrotic cells, Q2 = Late apoptotic cells, Q3 = Early apoptotic cells, Q4 = Live cells.

^{a-f} Values within a column with different superscript letters differ significantly ($p < 0.05$).

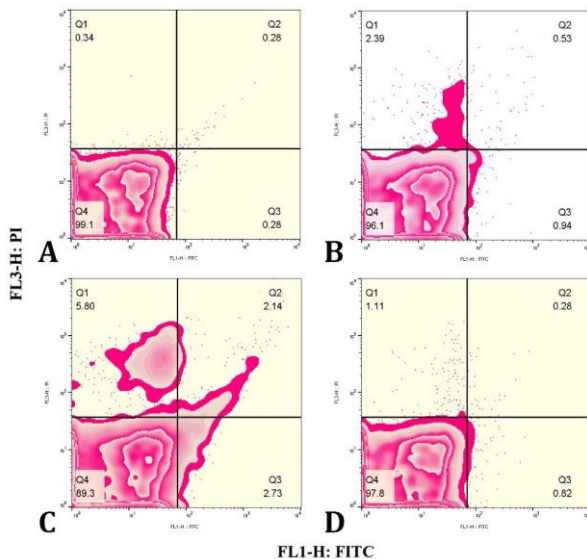


Fig. 2. Dot-plots of staining with Annexin-V and propidium iodide (PI; FL1: Annexin-V fluorescence, FL2: PI fluorescence) in thawed Simmental bull chilled spermatozoa treated with ammonium chloride (AC; 0.00 and 175 $\mu\text{M L}^{-1}$), alpha-ketoglutarate (AKG; 0.00, 200, 400 and 800 mM L^{-1}) and folic acid (FA; .00 and 5,000 nM L^{-1}). **A)** Gate area (unstained), **B)** 1 (control 1) = AKG 0.00 mM L^{-1} + FA 0.00 nM L^{-1} + AC 0.00 $\mu\text{M L}^{-1}$, **C)** 2 (control 2) = AKG 0.00 mM L^{-1} + FA 0.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$, and **D)** 9 = AKG 8.00 mM L^{-1} + FA 50.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$. Q1: Represents necrotic cells negative for Annexin-V but positive for PI (A-/PI+). Q2: Represents late apoptotic cells bound to both Annexin-V and PI (A+/PI+), Q3: Contains early apoptotic cells positive for Annexin-V but negative for PI (A+/PI-), Q4: Contains viable nonapoptotic cells negative for both Annexin-V and PI (A-/PI-). FITC: Fluorescein isothiocyanate.

Survival rate. The results of Eosin-Nigrosine staining are shown in Table 4. The groups 3 (92.90 \pm 1.21), 4 (93.16 \pm 1.29), 5 (94.75 \pm 1.13), 6 (93.21 \pm 1.49), 7 (91.53 \pm 1.22), 8 (93.10 \pm 0.18), and 9 (94.80 \pm 0.69) had increased values ($p < 0.05$) compared to the control group 2 (87.52 \pm 1.00).

Plasma membrane integrity. The results for hypo-osmotic swelling test of the sperm cells are shown in Table 4. All experimental groups showed higher ($p < 0.05$) hypo-osmotic swelling test values compared to the control group 2 (89.03 \pm 2.22).

DNA damage. The results of the Acridine Orange staining assay are shown in Table 4 and Figure 3. The results showed that the mean \pm SEM percent of DNA damage in all groups was significantly ($p < 0.05$) decreased compared to the control group 2 (7.78 \pm 0.84).

Table 4. Mean \pm SEM values of survival rate, plasma membrane integrity and DNA damage of Simmental bull chilled spermatozoa treated with ammonium chloride (AC; 0.00 and 175 $\mu\text{M L}^{-1}$), alpha-ketoglutarate (AKG; 0.00, 2.00, 4.00 and 8.00 mM L^{-1}) and folic acid (FA; 0.00 and 50.00 nM L^{-1}).

Groups	Survival rate	Membrane integrity	DNA damage
1	95.53 \pm 1.65 ^b	95.21 \pm 0.70 ^{bc}	1.92 \pm 0.38 ^d
2	87.52 \pm 1.00 ^a	89.03 \pm 2.22 ^a	7.78 \pm 0.84 ^a
3	92.90 \pm 1.21 ^{bc}	95.95 \pm 1.14 ^{bc}	4.64 \pm 0.54 ^b
4	93.16 \pm 1.29 ^{bc}	95.92 \pm 0.95 ^{bc}	3.94 \pm 0.85 ^{bc}
5	94.75 \pm 1.13 ^b	97.70 \pm 0.64 ^b	3.12 \pm 0.58 ^{cd}
6	93.21 \pm 1.49 ^{bc}	96.48 \pm 0.79 ^{bc}	2.96 \pm 0.59 ^{cd}
7	91.53 \pm 1.22 ^c	94.30 \pm 1.55 ^c	2.68 \pm 0.54 ^{cd}
8	93.10 \pm 1.18 ^{bc}	95.99 \pm 0.93 ^{bc}	2.57 \pm 0.53 ^d
9	94.80 \pm 0.69 ^b	96.94 \pm 0.69 ^{bc}	2.28 \pm 0.41 ^d

Group 1 (control 1) = AKG 0.00 mM L^{-1} + FA 0.00 nM L^{-1} + AC 0.00 $\mu\text{M L}^{-1}$; Group 2 (control 2) = AKG 0.00 mM L^{-1} + FA 0.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$; Group 3 = AKG 2.00 mM L^{-1} + FA 0.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$; Group 4 = AKG 4.00 mM L^{-1} + FA 0.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$; Group 5 = AKG 8.00 mM L^{-1} + FA 0.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$; Group 6 = AKG 0.00 mM L^{-1} + FA 50.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$; Group 7 = AKG 2.00 mM L^{-1} + FA 50.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$; Group 8 = AKG 4.00 mM L^{-1} + FA 50.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$; Group 9 = AKG 8.00 mM L^{-1} + FA 50.00 nM L^{-1} + AC 175 $\mu\text{M L}^{-1}$.

^{a-d} Values within a column with different superscript letters differ significantly ($p < 0.05$).

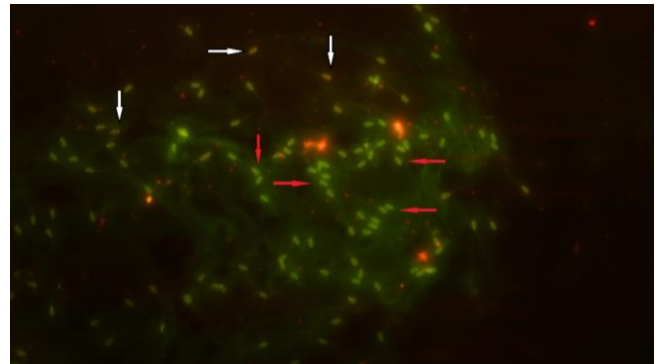


Fig. 3. Simmental bull sperm stained by Acridine Orange. The cells with damaged DNA appear yellow to orange (red arrows), and the cells with undamaged DNA appear green (white arrows).

Discussion

The blood concentration threshold for the occurrence of clinical signs of ammonia toxicity varies among different species, ranging from 0.70 mg dL⁻¹ in cattle,²⁶ to 30.00 µM L⁻¹ in humans. The results of other studies have shown that the normal concentration of cattle blood ammonia ranges from 7.00 to 95.00 µM L⁻¹.²⁷ Interestingly, in humans, an ammonia concentration three to five times higher than the normal limit is not associated with clinical signs.²⁸ The findings of the present study also revealed a significant gap between the threshold for clinical symptoms and the cytotoxic levels of ammonia in bulls. The results of this study demonstrated the ameliorative effect of AKG and FA on negative influence of ammonia on sperm motility *in vitro*. The results of the present research were consistent with another study that demonstrated the negative impact of AC on the kinematic motility of mammalian sperm. It is postulated that ammonia might hinder adenosine triphosphate production in sperm by inhibiting adenosine triphosphatase activity.⁵ Also, sperm metabolic pathways heavily depend on the host animal species. For instance, glycolysis is the dominant energy supply pathway in humans and mice, while horses rely on oxidative phosphorylation, and cows utilize a combination of glycolysis and oxidative phosphorylation pathways.²⁹ The AKG, belonging to the ketoacid group, naturally constitutes approximately 4.54% of ketoacid derivatives in bull seminal plasma. It plays a pivotal role as an intermediary metabolite within the tricarboxylic acid cycle, also known as the Krebs cycle or citric acid cycle. Additionally, AKG contributes to extended lifespan, stem cell potential modulation, and delayed age-related fertility decline.³⁰ Recent findings have indicated that calcium-AKG enhances lifespan by reducing systemic inflammatory cytokine concentrations and promoting anti-inflammatory cytokines.³¹ It also plays a key role in reactive oxygen species (ROS) detoxification and serves as a crucial component of the oxidative defense system.³² The flow cytometry and DNA damage results of this study align with previous research, indicating AKG capacity to counteract ammonia effects on sperm cells. The AKG mitigates apoptosis in granulosa cells of the mouse ovary, enhancing glycolysis rate-limiting enzyme expression.³³ The results of this study demonstrated that the antioxidants employed had a statistically significant effect on the enzyme activity concentrations of CAT, SOD, and TAC in controlling ammonia-induced damage to sperm. The ammonia toxicity can elevate ROS concentrations, the expression of antioxidant enzymes (SOD and GPx), and decreases cell survival and reduced expression of anti-apoptotic factors (Bcl-2).³⁴ Existing evidence suggests that both enzymatic and nonenzymatic antioxidants can be employed to mitigate excessive ROS-induced damage in sperm, particularly antioxidants specifically targeting mitochondria.³⁵ An

imbalance between antioxidant defense mechanisms within cells can lead to reduced survival rates, decreased motility, increased DNA damage, morphological defects, and lipid peroxidation, ultimately culminating in cell apoptosis.³⁶ Hyperammonemia, resulting from liver damage, oxidative DNA damage, and impaired adenosine triphosphate production along with energy depletion, poses a significant risk factor for brain abnormalities.³⁷ The AKG capacity to boost antioxidant function by increasing glutamine content and enhancing antioxidant systems is well documented.³⁸ Furthermore, AKG significantly elevated SOD activity while reducing malondialdehyde concentrations, signifying improved intestinal antioxidant capacity.³⁹ Folic acid is a synthetic, oxidized version of the natural water-soluble vitamin B9, which is folate. Seminal plasma folates include 5-methyl tetrahydrofolate and non-methyl tetrahydrofolates. Among these, 5-methyltetrahydrofolate is the active form of folate in the body. Furthermore, the concentration of non-methyl tetrahydrofolates in seminal plasma correlates significantly with sperm density and total sperm count.⁴⁰ The primary reason for increased sperm count following oral administration of FA, combined with other supplements such as vitamin E (400 IU *per day*) and selenium (60.00 mg *per day*), is the suppression of the urea cycle without affecting ammonia recycling, leading to improved sperm motility.⁴¹ Similarly, characteristics of epididymal sperm include enhanced individual mobility and forward movement, reduced abnormal activity (backward, vibration, rotation), and a decreased percentage of dead and abnormal sperm following FA treatment.⁴² The application of FA in male infertility treatment stems from its ability to scavenge free radicals.⁴³ Recent research reports demonstrated that administration of FA combined with zinc sulfate,⁴⁴ vitamin E and selenium,⁴⁵ significantly improved sperm parameters.⁴⁶ There exists a negative and significant correlation between FA concentration in semen and the DNA fragmentation index of human sperm.⁴⁷ Furthermore, FA deficiency has been associated with increased apoptosis and genome breakage in testicular tissue.⁴⁸ In mammals, there are an array of antioxidant defense mechanisms to protect vital biomolecules against oxidative damage. The well-known of these agents are SOD, CAT, GPx, glutathione, vitamin C, and vitamin E, which effectively neutralize most ROS.⁴⁹ *In vitro* studies have revealed that ammonia amplifies ROS production, resulting in elevated superoxide, lipid peroxidation, and xanthine oxidase concentrations in the brains of mice with acute hepatitis encephalitis. These changes are accompanied by reduced ROS protection, low glutathione concentrations, and diminished expression and activity of CAT, GPx, and SOD.⁵⁰ Recent studies have consistently shown that AKG and FA can enhance antioxidant function against oxidative stress.^{51,52} These

findings suggest that future research will expand our understanding of the cellular and molecular mechanisms and diverse effects of AKG and FA as promising antioxidants, ultimately yielding positive outcomes for ammonia control. Consequently, these insights can potentially pave the way for clinical applications to promote health. Nevertheless, further research is warranted to determine the optimal doses, forms, and administration routes of AKG and FA. Given the close connection between the accumulation of nitrates in edible plant organs and the usage of nitrogen fertilizers, there is a continuous risk for ruminants due to the presence of nonprotein nitrogen in plants. Despite the advantages of AKG and FA, several challenges and drawbacks exist when utilizing them. Both inadequate and excessive doses of AKG and FA as well as repeated use, might diminish their effectiveness and lead to adverse or even hazardous effects. An essential point to note is that different doses of AKG may be needed to achieve maximal benefits, potentially showing reproduction-dependent effects. Frequent high-dose AKG supplementation may also carry adverse side effects. Furthermore, it might contribute to the development of certain cancers through various cellular and molecular mechanisms such as activating the cell cycle and generating glucose and glutamine principal energy sources for cancer cells. In the case of FA, further research is essential to comprehensively understand its effects and potential limitations.

In conclusion, this study indicated that supplementation with AKG and FA at the desired concentrations counteracted the adverse effects of ammonia toxicity that precede clinical signs. The concentrations that exhibited the most favourable outcomes were 4.00 and 8.00 mM L⁻¹ AKG combined with 50.00 nM L⁻¹ FA. Further investigations may be required to explore the potential of higher concentrations of FA and AKG, along with other antioxidants, to counteract the detrimental impact of ammonia on bull sperm cells. It is noteworthy that further studies are needed to evaluate the fertility of these sperms either *in vitro* or *in vivo*.

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Conflicts of interest

The authors declare no conflict of interest.

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