

## Effects of an allostatic modulator on the behavior and blood indicators of young bulls after transport

Maria Salud Rubio Lozano<sup>1\*</sup>  
Rubén Danilo Méndez Medina<sup>2</sup>  
Karla Reyes Mayorga<sup>1</sup>  
Maria Elena Rubio García<sup>1</sup>  
Tania Manu Ngapo<sup>3</sup>  
Karen F. Mancera<sup>4</sup>  
Francisco Aurelio Galindo Maldonado<sup>4</sup>

<sup>1</sup>Laboratorio de Ciencia de la Carne, Centro de Enseñanza Práctica e Investigación en Producción y Salud Animal, Facultad de Medicina Veterinaria y Zootecnia, Cruz Blanca 486, San Miguel Topilejo, Mexico DF 14500, Mexico.

<sup>2</sup>Departamento de Patología, Facultad de Medicina Veterinaria y Zootecnia, Avenida Universidad 3000, Circuito exterior, Ciudad Universitaria, Del. Coyoacán, Mexico DF 04150, Mexico.

<sup>3</sup>Food Research and Development Centre, Agriculture and Agri-Food Canada, 3600 boul. Casavant Ouest, Saint-Hyacinthe, Québec, J2S 8E3, Canada.

<sup>4</sup>Departamento de Etología, Fauna Silvestre y Animales de Laboratorio, Facultad de Medicina Veterinaria y Zootecnia, Avenida Universidad 3000, Circuito exterior, Ciudad Universitaria, Del. Coyoacán, Mexico DF 04150, Mexico.

\*Corresponding author:  
Tel: +52 55 37336720  
Email address:  
[msalud65@gmail.com](mailto:msalud65@gmail.com)

### Abstract

An allostatic modulator (AM) composed of ascorbic acid, acetylsalicylic acid, sodium chloride and potassium chloride was dissolved in the drinking water of three groups of young bulls ( $n = 7$ ) and administered to them for 7 d after 20.42 h of transport (Control (C) = 0 g/head/d, G2 = 30 g/head/d, G3 = 60 g/head/d). The effects of the AM on behavior and blood cellular and biochemical components were measured. G3 animals spent less time lying and more time standing than C animals ( $P = 0.001$  and  $P = 0.02$ , respectively), indicating less fatigue. G3 bulls presented a lower frequency of agonistic interactions than those in the G2 group ( $P = 0.001$ ), suggesting a possible effect on aggression that warrants further evaluation. The AM affected creatinine kinase (CK) activity in the G2 group compared to the C group ( $P = 0.04$ ), suggesting an effect of AM components that has not been previously reported. Cortisol levels remained unaffected by AM supplementation ( $P = 0.55$ ). These preliminary findings suggest an effect of supplementing cattle with an AM after long-haul transport.

**Keywords:** allostasis; animal behavior; blood stress indicators; beef

Accepted: 2018-09-28  
Published: 2019-02-05

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can be found on page 9

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### Cite this as:

Rubio Lozano MS, Méndez Medina RD, Reyes Mayorga K, Rubio García ME, Ngapo TM, Mancera KF, Galindo Maldonado FA. Effects of an allostatic modulator on the behavior and blood indicators of young bulls after transport. *Veterinaria México OA*. 2018;5(4). doi: 10.22201/fmvz.24486760e.2018.4.539

## Introduction

Animal welfare concerns associated with transport stress include limited access to feed and water, variable climatic conditions, noise, vibrations, poor handling and social stress.<sup>1-4</sup> Physiological effects include hypoglycemia, dehydration, energy depletion, protein degradation, electrolyte imbalance and stress response activation.<sup>5,6</sup>

Allostasis is the readjustment of requirements in response to stress to maintain an optimal physiological state.<sup>7</sup> An allostatic modulator (AM) regulates the physiological stress response to maintain allostasis.<sup>8</sup> Several AM substances have been developed, such as dopaminergic antagonists<sup>9</sup> and  $\beta$ -adrenergic receptor blockers.<sup>10</sup> In addition, vitamins,<sup>11-13</sup> microelements,<sup>14,15</sup> and amino acids<sup>13</sup> have also been tested. Improvements have been observed recently in blood indicators and meat color parameters in 18- to 20-month-old bulls fed with 10 g/head/d of an AM composed of acetylsalicylic acid, ascorbic acid, sodium chloride and potassium chloride for 30 d preslaughter.<sup>16</sup>

Acetylsalicylic acid inhibits the cyclooxygenase pathway,<sup>17</sup> contributing to the regulation of ACTH release<sup>18</sup> and reducing stress. Additionally, Pardue<sup>19</sup> has proposed that ascorbic acid might suppress adrenocortical steroidogenesis. Finally, electrolyte administration before slaughter stabilizes serum components.<sup>20</sup> We performed a preliminary evaluation of how the aforementioned AM diluted in the drinking water of young bulls for 7 d after long-haul transport affected the bulls' physiological stress response.

## Materials and methods

Animal use was regulated by the Subcomité Interno para el Cuidado y Uso de los Animales para Experimentación of the Facultad de Medicina Veterinaria y Zootecnia (SICUAE - FMVZ) at the Universidad Nacional Autónoma de México (UNAM).

## Animals

Seventy-seven commercial crosses of 16- to 18-month-old bulls (*Bos indicus* (Brahman and Nellore) and *Bos taurus* (Charolais, Swiss Brown and Simmental)) with an average weight of  $335 \pm 34$  kg (mean  $\pm$  SE) were held for 3 h in holding pens at a breeding farm in the state of Campeche, Mexico, where they were also raised. Animals showed a calm temperament, and they responded well to human interaction. In the summer (mean temperature of 34.5°C), bulls were loaded onto a commercial cattle truck (2.7 m wide x 18 m long trailer, six compartments, anti-slip flooring and roof) in groups of 10-14 animals per compartment without mixing unknown individuals. Bulls were transported 1100 km on paved roads to a feedlot cattle ranch in the state of Veracruz, Mexico. The trip lasted 20.42 h, including a 9-h waiting period inside the truck for customs. Bulls had no access to food or water during transport. Upon arrival, animals were unloaded by three operators using a concrete ramp to a holding pen using either electric prods (54 animals) or a wooden stick (46 animals).

Once in the holding pen, 21 animals were randomly chosen and divided into three groups of seven, which were directed to different pens through 0.85-m-wide

alleyways of approximately 3 m in length using electric prods (15 animals) or a wooden stick (6 animals). Feedlot pens were 30 m x 20 m with a 20 m long feeder of approximately 0.5 m diameter, a 3 m x 1 m x 0.9 m water trough, soil floors and a 3-m-high metal roof (30% shadow). During experimentation, feed composed of 45% high-quality hay and 55% concentrates (sorghum, corn and soybean plus vitamin and mineral premixes) was provided *ad libitum*.

### Treatments

Groups received different doses of AM: Control (C) = 0 g/head/d, G2 = 30 g/head/d and G3 = 60 g/head/d. The AM was composed of acetylsalicylic acid (140 g), ascorbic acid (100 g), sodium chloride (128 g) and potassium chloride (128 g). After calculating the volume of water/head/pen (10% of the animal's body weight<sup>21</sup>), AM was diluted in 100 L of water (210 g/100 L for G2 and 420 g/100 L for G3). Once diluted, AM dilutions or water were placed in the corresponding drinking troughs for *ad libitum* consumption immediately after placement into pens. Since the experiment was carried out under commercial conditions, the amount of water consumed per animal was not possible to calculate; therefore, it was assumed that the animals' daily water intake was the average reported in other studies assessing this variable.<sup>22</sup> Drinking troughs of all groups were cleaned and filled with new dilutions or water (C) daily. Animals were subjected to treatments for 7 d immediately after transport.

### Behavioral assessment

Ethograms were determined during an *ad libitum* sampling.<sup>23</sup> Behavior was assessed for 6 h daily (1100 to 1700 h) beginning the day animals were placed into pens to receive water treatments during a 7-d period. Three different observers placed at 10 m from the pen (one observer per pen, observers rotating among pens, interobserver reliability:  $R=0.95$ ,  $P < 0.05$ ) carried out observations. Individual behavior was evaluated using scan sampling every 10 min, and focal sampling was used to obtain frequencies of social interactions ( $5.5 \pm 0.19$  h (mean  $\pm$  SE) of focal observation/animal).<sup>23</sup> Individual behaviors recorded were walking, standing, lying, drinking (head in water trough) and eating (head in feeding trough). Social interactions recorded were agonistic (head-butts to head and/or flank of a pen-mate) and affiliative (licking and/or sniffing any body part of a pen-mate). After data collection, proportions of time of individual behaviors were obtained by calculating the number of scans for each behavior divided by total scans, whereas the frequencies of social interactions were obtained by calculating the number of interactions divided by hours of observation/animal.<sup>23</sup>

### Blood sample collection and analyses of cellular components and biochemical indicators

Blood samples were taken on day 1 (D1, before AM administration), D3, D5 and D7. Using a squeeze chute, four 5-ml blood samples from the tail vein were collected into sterile vacutainer tubes. Three tubes contained ethylenediaminetetraacetic acid (EDTA) and were stored at 4°C. Afterwards, total leukocyte, neutrophil, eosin-

ophil and lymphocyte counts were obtained according to Núñez and Bouda.<sup>24</sup> The neutrophil:lymphocyte ratio (N:L) was also calculated.

EDTA-free tubes were centrifuged at 2500 rpm for 10 min at room temperature. After centrifugation, serum was collected into 5-ml sterile vials and stored at 0°C. Plasma glucose concentrations were determined using the GOD-PAP test without deproteinization (GL 2623, RANDOX®). Creatine kinase activity (CK) was determined by the CK-NAC activated method (RANDOX®). Aspartate aminotransferase (AST) activity was measured by the GOT method (RANDOX®). The lactate concentration was determined by hydrogen peroxide production during lactate to pyruvate transformation (RANDOX® Manual RXMONZA). Plasma cortisol (µg/dl) was determined using a competitive immunoassay technique.<sup>25</sup> Plates were read using a Vitalab 10 spectrophotometer (Vital Scientific, Dieren, the Netherlands) at 505 nm for glucose; 340 nm for CK, AST and lactate; and 410 nm for cortisol. The evaluation of glucose, CK, lactate and plasma cortisol concentrations and the assessment of AST activity were performed using reagents previously validated in a prior study performed by this group.<sup>16</sup>

### Statistical analysis

Differences in proportions of time of individual behavior and frequencies of social interactions by treatment were tested using the Kruskal-Wallis test (KW). If significant ( $P < 0.05$ ), pairs of mean ranks were compared using the Dunn-Bonferroni (DB) test adjusted with the Bonferroni correction. A linear effects mixed model (LEM) with 'AM dose', 'sampling day', and 'AM dose\*sampling day' as factors and 'bull' as a random factor was established to evaluate the effects in blood cellular components and biochemical indicators. F-values were approximated using the Kenward-Roger procedure as a standard SPSS method, thereby obtaining non-integer values for the denominator degrees of freedom. Residuals were tested for normal distribution with the Shapiro-Wilk test, and if not normally distributed ( $P \leq 0.05$ ), data were transformed using square root or logarithm 10, whichever most effectively returned residuals to a normal distribution. When LEM results were significant, a post hoc Bonferroni test was used to compare means ( $P \leq 0.05$ ). If residuals were not normal after transformation, values were analyzed with KW as explained above. Calculations were performed with the program IBM SPSS statistics, version 20.

## Results and discussion

### AM effects on animal behavior

Despite the presence of confounding factors, such as possible behavioral differences between sampling and nonsampling days, which were not estimated due to the observational methodology that we were able to perform under commercial conditions, these results suggest an effect of the allostatic modulator on behavior and physiology that needs to be further evaluated in better controlled conditions.

Time standing was greater in G3 than in C, with G2 being the intermediate, and the lying time was shorter in G3 than in C. G3 also had the highest frequency of behavioral events. The frequency of agonistic interactions was higher in G2 and

significantly lower in G3, with C being the intermediate (Table 1). Fifteen hours of transport has been shown to produce less standing time in cattle than 2.7 h of transport.<sup>26</sup> The longer standing and shorter lying times in G3 suggest faster recovery from transport stress with 60 g/head/d of AM. Fewer agonistic interactions in G3 than in G2 suggest less agitated animals with the highest AM dose, as some of the AM components, such as acetylsalicylic acid and ascorbic acid, have been shown to decrease stress-related behavior in lambs<sup>27</sup> and goats.<sup>28</sup> However, the results are unclear because G3 was not different from C. Further research is needed to clarify the possible benefits of AM supplementation on behavior.

### *Effect of AM on blood components*

Leukocytes were positively correlated with neutrophils ( $\rho=0.79$ ,  $P < 0.001$ ) and lymphocytes ( $\rho=0.69$ ,  $P = 0.001$ ). The eosinophil count remained unaffected ( $\chi^2_{DF} = 3.222_2$ ,  $P_{KW} = 0.2$ ). No significant differences between treatments or treatment by sampling days were found for blood cellular components, although leukocyte and neutrophil count and the N:L ratio changed across sampling days (Table 2).

Changes in cell counts over time are related to stress adaptation.<sup>29,30</sup> Correlations between leukocyte, neutrophil and lymphocyte counts, as well as the release of catecholamines during stress, have been previously found.<sup>31,32</sup> The AM had no effects on cellular components, despite previous studies in which ascorbic acid administered 12 h before transport helped maintain blood cellular counts close to prestress levels in goats.<sup>33</sup> In our study, cattle received AM after transport, which could have made a difference in AM efficacy. Further research testing AM supplementation at different times is necessary.

### *Effects of AM on biochemical serum indicators*

CK activity was higher in C than in G2, with G3 being the intermediate ( $P = 0.04$ ). No differences in the other indicators were observed with AM supplementation. Glucose concentration was higher on D1 than on the rest of the days ( $P = 0.0001$ ,  $F_{NDF, DDF} = 22.48_{3, 54}$ ; D1 = 6.19 mmol/L, D3 = 4.57 mmol/L, D5 = 3.905 mmol/L, D7 = 4.67 mmol/L). CK activity was higher on D1 than on D5 and D7, with the lowest activity on D5 ( $P = 0.001$ ,  $F_{NDF, DDF} = 6.68_{3, 52.41}$ ; D1 = 353.8 U/L, D3 = 262.6 U/L, D5 = 129.01 U/L, D7 = 161.929 U/L). The sampling day affected AST activity, as the values were significantly higher on D1 and D2 than on D5 and D7 ( $\chi^2_{DF} = 29.64_3$ ,  $P_{KW} < 0.001$ ; mean ranks: D1 = 62.07 U/L, D3 = 50.71 U/L, D5 = 27.52 U/L, D7 = 29.69 U/L).

CK activity relates to muscle damage<sup>34</sup> and increases after transport in a positive correlation with transport time.<sup>30</sup> To date, there is no information on the effects of these AM components on CK activity after transport; our study suggests that 30 g/head/d AM reduces CK activity in cattle. However, as Ck activity did not differ between the highest AM dose and the C, further research is needed to understand the effects of different doses on this biochemical marker.

Glucose tendencies over time relate to the initial gluconeogenesis related to stress and its eventual return to normal concentrations.<sup>35-37</sup> CK and AST activities also decrease over time after transport stress.<sup>38</sup> For cortisol, while no effects have

**Table 1.** Proportions of time of individual behaviours and frequency of social interactions for bulls receiving different doses of an Allostatic Modulator (AM) contrasted with Kruskal-Wallis test ( $KW$ ). Pairs of ranks were contrasted using the Dunn-Bonferroni tests ( $DB$ ).  $\chi^2_{DF}$  = Chi squared value Degrees of Freedom.  $P_{KW}$  = P value for the Kruskal-Wallis test  $P_{DB}$  = P value for the Dunn-Bonferroni tests with Bonferroni correction. Standard Error of the Mean = SEM. Means that do not share a letter are significantly different ( $P < 0.05$ ).

Behaviour	Treatments (AM dose)			SEM	$\chi^2_{DF}$ (PKW) Treatments	Z (PDB) G1 vs G2	Z (PDB) G1 vs G3	Z (PDB) G3 vs G2
	C (g/head/day)	G2 (30 g/head/day)	G3 (60 g/head/day)					
Eating (mean rank of proportion of time)	10	13	10	-	4.2 <sub>2</sub> (0.12)	-	-	-
(% proportion of time)	18.70	21.28	22.91	0.21	-	-	-	-
Drinking (mean rank of proportion of time)	10.07	11.36	11.57	-	0.24 <sub>2</sub> (0.89)	-	-	-
(% proportion of time)	1.51	1.69	1.64	0.29	-	-	-	-
Walking (median of proportion of time)	11.14	9.29	12.57	-	0.99 <sub>2</sub> (0.607)	-	-	-
(% proportion of time)	3.21	2.61	3.63	0.65	-	-	-	-
Standing (mean rank of proportion of time)	5.71 <sup>a</sup>	12.57 <sup>ab</sup>	14.71 <sup>b</sup>	-	8.04 <sub>2</sub> (0.02)	-2.07 (0.12)	-2.71 (0.02)	-0.65 (0.9)
(% proportion of time)	15.25	20.43	25.80	0.22	-	-	-	-
Lying (mean rank of proportion of time)	17.43 <sup>a</sup>	9.86 <sup>ab</sup>	5.71 <sup>b</sup>	-	12.831 <sub>2</sub> (0.002)	2.28 (0.067)	3.53 (0.001)	1.25 (0.64)
(% proportion of time)	53.39	41.11	35.75	0.27	-	-	-	-
Behavioural events (mean rank of events/h)	11.29 <sup>ab</sup>	4.29 <sup>a</sup>	17.43 <sup>b</sup>	-	15.75 <sub>2</sub> (0.0001)	2.11 (0.104)	-1.85 (0.2)	-3.97 (0.0001)
(events/h)	4.01	2.25	7.48	0.73	-	-	-	-
Agonistic interactions (mean rank of interactions/h)	10.36 <sup>ab</sup>	17.41 <sup>a</sup>	5.50 <sup>b</sup>	-	12.94 <sub>2</sub> (0.002)	-2.09 (0.11)	1.49 (0.4)	3.58 (0.001)
(events/h)	0.32	0.87	0.05	0.11	-	-	-	-
Affiliative interactions (mean rank of interactions/h)	14.21	10.64	8.14	-	4.45 <sub>2</sub> (0.108)	-	-	-
(events/h)	0.13	0.11	0.03	0.05	-	-	-	-

**Table 2.** Blood cellular and biochemical components of bulls administered with three different doses of AM contrasted by AM dose and sampling day (D1, D3, D5, and D7) SED = Standard Error of the Difference. F = F value. NDF, DDF = Numerator Degrees of Freedom, Denominator Degrees of Freedom.

BLOOD COMPONENT	MEANS												SED	F <sup>NDF, DDF</sup> (P VALUE)		
	C (0 g/head/day)				G2 (30 g/head/day)				G3 (60 g/head/day)					AM dose	Sampling day	AM dose * Sampling day
	D1	D3	D5	D7	D1	D3	D5	D7	D1	D3	D5	D7				
<b>Cellular</b>																
Leukocytes (x 10 <sup>9</sup> /L)	15.14	11.14	11.57	9.43	16.00	14.43	10.71	10.29	16.57	14.00	13.29	10.00	1.140	1.83 <sub>1,18</sub> (0.19)	15.48 <sub>3,54</sub> (0.0001)	0.74 <sub>6,54</sub> (0.59)
Neutrophils (x 10 <sup>9</sup> /L)	6.43	4.14	4.00	3.14	6.57	5.29	3.00	2.86	8.57	5.71	4.29	3.00	0.715	1.38 <sub>2,18</sub> (0.28)	26.31 <sub>3,54</sub> (0.0001)	1.16 <sub>6,54</sub> (0.34)
Lymphocytes (x 10 <sup>9</sup> /L)	7.86	6.57	7.29	5.43	7.71	8.57	6.71	6.57	7.14	6.71	8.43	6.43	0.961	0.35 <sub>2,18</sub> (0.71)	1.52 <sub>3,54</sub> (0.22)	0.83 <sub>6,54</sub> (0.55)
N:L ratio	0.98	0.66	0.63	0.64	0.74	0.63	0.45	0.48	1.22	0.72	0.53	0.607	0.130	1.14 <sub>2,17,79</sub> (0.34)	11.92 <sub>3,52,09</sub> (0.0001)	1.09 <sub>6,52,08</sub> (0.38)
<b>Biochemical</b>																
Glucose (mmol/L)	5.86	4.28	3.14	4.28	6.57	4.57	4.57	4.43	6.14	4.85	4.00	5.29	0.433	1.54 <sub>2,18</sub> (0.24)	22.48 <sub>3,54</sub> (0.0001)	1.17 <sub>6,54</sub> (0.34)
CK (log <sub>10</sub> [U/L + 1])	2.68	2.82	2.07	2.31	2.41	2.13	2.15	2.05	2.55	2.32	2.13	2.27	0.136	3.91 <sub>2,17,61</sub> (0.04)	6.68 <sub>3,52,41</sub> (0.001)	1.62 <sub>6,52,35</sub> (0.16)
(U/L)	477.63	659.69	116.49	203.17	257.04	133.89	140.25	111.202	353.81	207.93	133.89	185.21	-	-	-	-
Lactate (mmol/L)	8.07	6.11	6.69	7.29	9.21	6.79	7.27	9.83	9.04	6.43	8.09	8.14	1.359	0.51 <sub>2,19,71</sub> (0.61)	2.32 <sub>3,41,17</sub> (0.89)	0.28 <sub>6,41,06</sub> (0.94)
Cortisol (√µg/dL)	1.54	2.06	1.53	2.02	1.77	1.92	1.82	1.34	2.17	1.88	1.83	2.01	0.254	0.602 <sub>2,17,22</sub> (0.55)	0.58 <sub>3,50,86</sub> (0.63)	1.33 <sub>6,50,81</sub> (0.26)
(µg/dL)	2.37	4.24	2.34	4.08	3.13	3.69	3.31	1.79	4.71	3.53	3.35	4.04	-	-	-	-

been reported in relation to road travel,<sup>30</sup> as observed in this study, other studies have shown increases<sup>35-39</sup> or decreases<sup>37</sup> in cortisol levels shortly after transport. Thus, further research is needed to understand cortisol activity after transport stress and the effects of AM supplementation.

## Conclusions

This experiment was carried out under commercial conditions, and it was not possible to separate transport from handling stress, nor the stress associated with the use of prods or wooden sticks for individuals; however, our aim was to evaluate the efficiency of the AM on animals, regardless of the source of stress. Likewise, the conditions in which this experiment was carried out did not enable us to evaluate the amount of water consumed per animal, and it was assumed that animals' daily water intake was the average reported in other studies assessing this variable.<sup>22</sup> Despite these and other possible cofounding factors associated with the methodological features of this study, it was observed that an AM dose of 60 g/head/d increased the average proportion of standing time and decreased the average proportion of lying time, suggesting that AM treatment could be associated with faster recovery rates after transport. The behavioral changes observed in this study need to be evaluated over time in further studies. For blood parameters, CK activity decreased with the lower but not the highest AM dose, suggesting a degree of effect on transport stress. Cortisol remained unaffected; however, as cortisol activity post transport has been reported to be highly variable, AM effects remain unclear for this marker and need to be explored in depth in future studies. Overall, this preliminary study suggests that AM administration for 7 d after 20.42 h of transport can affect the behavior and blood components of cattle. Subsequent studies need to consider greater sample sizes, different methods of AM administration in which water ingestion per animal can be measured and behavioral evaluations that include dominance and hierarchies within groups as covariates.

## Acknowledgements

This research was funded by the National Council of Science and Technology (CONACyT-Atisa, No. 17549-1590-15-X-05). We greatly appreciate the support given by the Pathology Department at the FMVZ (UNAM) for the stress blood indicator analysis. This contribution is also part of Karen F. Mancera's postdoctoral project, supported by the Postdoctoral Scholarship Program UNAM-DGAPA at the Faculty of Veterinary Medicine - UNAM.

## Conflicts of interest

The authors declare no conflicts of interest.

## Author contributions

MSRL: project design, data analysis and interpretation, manuscript development.

RDMM: project design and leading, experimental work.

KRM: experimental work.

MERG: contribution to analysis techniques, contribution to manuscript development.

TMN: data analysis and interpretation, contributions to manuscript development.

KM: data analysis and interpretation, contribution to manuscript development.

FAGM: data analysis and interpretation, contribution to manuscript development.

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