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Behavioral Changes Related to Zinc Deficiency in Buffalo Calves (*Bubalus bubalis*)

Ibrahim M. Hegab[1*] and Walaa I. Mohamaden[2]

[1]Department of Behavior & Management of Animal, Poultry and Fish, Faculty of Veterinary Medicine, Suez Canal University, Ismailia 41522, Egypt.
[2]Department of Animal Medicine, Faculty of Veterinary Medicine, Suez Canal University, Ismailia 41522, Egypt.
***Corresponding author:** ibrahim_hegab@vet.suez.edu.eg

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ABSTRACT

Mineral deficiencies are a prevalent challenge for livestock producers and veterinarians. Twenty-five weaned buffalo calves of 90-115 days old and 85-135kg body weight were used in the current study. Calves were clinically examined, and 13 calves showed abnormal hair coat conditions (Zinc deficient, G1) while the other 12 calves were clinically healthy (Apparently healthy, G2). Blood samples were collected from each individual animal for assessment of the blood zinc and copper levels by flameless atomic absorption spectrophotometer and for complete hematological analysis. Three behavioral tests were used to assess the anxiety and fear reactions toward novel circumstances: the open field (OFT), the novel object (NOT) and the novel human (NHT) tests. Our results showed that the average body weight did not significantly differ between groups although G1 group showed lower average body weight than G2. The blood zinc level was significantly ($P<0.05$) lower in G1 than G2, but the copper level did not significantly vary between groups. Mean corpuscular volume was significantly increased in G1 than G2 while mean corpuscular hemoglobin concentration was significantly reduced. Calves in G1 displayed distinct anxiety and fear related behavioral responses toward novel places (OFT), object (NOT) and human (NHT). We concluded that these early behavioral changes in buffalo calves are related to the developmental deficiency in zinc levels. Our study highlighted the functional significance of zinc in early behavior development and skin disorders in buffalo calves.

Key words: Buffaloes, Calves, Behavior, Zinc, Deficiency.

INTRODUCTION

Living organisms need minerals as well as an essential nutritional element to continue their normal development which plays a crucial role in optimal growth and cognitive functions (Saleh 2019). Trace minerals such as zinc and copper are crucial for the survival of animals since they are involved in various biological, chemical and metabolic processes. In addition, they are vital co-factors for different pathways in the immune system, activations of enzymes and a stabilizer for secondary molecular structures. Zinc stabilizes the DNA structure and facilitates its replication (Youssef et al. 2022). Substantial studies have revealed that supplementation with trace minerals such as zinc enhances the growth performance and immunity of calves (Wo et al. 2022). The same trend was also observed for copper that a study showed that an improved growth following subcutaneous injection of copper ethylenediamine tetra acetate, and the treated calves had a 2.8% increase in adjusted weaning weights (Naylor et al. 1989). This might emphasize the importance of trace minerals during the early life stages in ruminants.

Optimum nutrition and management have valuable effects on the prospective productivity in calves, especially in the early developmental period. Ruminants are often suffering from deficiency of the trace elements such as: zinc, cobalt, manganese, selenium and copper due to improper management or dietary programs, nutrients interactions and/or other factors related to the animal or its environment (Saleh 2019). Mineral deficiency can alter or even halt metabolic and biochemical pathways necessary for normal body functions which results in appearance of clinical symptoms of various aspects and intensities. For example, calves suffer from mineral deficiency have been found to be more susceptible to other diseases especially bovine respiratory disease, and they often show growth retardation (Bailey et al. 2015). Mineral deficiency in

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animals is also associated with poor hair coat, skin lesions, white patches or depigmentation of hair and hair loss (Hill and Shannon 2019). Further complications might develop such as delayed wound healing, cell-mediated immune dysfunction, and abnormal neurosensory changes (Prasad 2013). On the other hand, body weight gain and feed conversion ratio were improved in zinc-supplemented calves alongside higher hemoglobin concentration, packed cell volume, total erythrocyte count, total leukocyte count, neutrophil and lymphocyte values compared to the control group (Kumar et al. 2018).

Let alone the metabolic and productive disorders, mineral deficiency has been implicated to decline in cognitive functions. Previous studies on experimental animals indicates that zinc deficiency during the early life stage can critically hamper brain function and behavior, in addition to brain development which finally can impair various aspects of animal cognitive development and interaction to the surrounding environment (Keller et al. 2001). Studies on the behavioral changes in zinc deficient animals yield contradictory results. On one hand, lethargy (reduced activity and responsiveness) was a characteristic sign in animals suffering from zinc deficiency (Golub et al. 1995). In contrast, zinc supplementation improves neuropsychological behavior and memory performance in different tasks and ages including short- and long-term recognition memory in young rats and short-term recognition memory in adult rats, and spatial working memory in adult rats (Sandusky-Beltran et al. 2017) which suggests that zinc function is linked to mental function (Takeda et al. 2006).

Buffaloes (*Bubalus bubalis*) are considered important agricultural livestock in Egypt and worldwide because these animals are the main source for superior quality animal proteins. Also, buffaloes have high disease resistance and thermal tolerance to hot climatic conditions and can convert poor roughages into meat and milk efficiently. Mineral deficiencies in buffaloes had been investigated in different studies (Kumar and Sharma 2007; Neto et al. 2007; Sharma et al. 2008; Mudgal et al. 2012), but to the best of our knowledge, very few studies have discussed the effect of mineral deficiency on the behavioral responses and cognitive performance during the early developmental stage in buffaloes. In the present study, the early behavioral and cognitive changes in buffalo calves showing skin lesion due to zinc deficiency was analyzed by three behavioral paradigms: the open field, the novel object and the novel human tests compared to calves showing normal skin coat. Also, the full hematological picture was also investigated.

MATERIALS AND METHODS

Animals and Clinical Examination

This experiment was performed at the animal educational and research farm, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt. All experimental procedures were approved, prior to the commencement of work, by the animal ethics committee of the Faculty of Veterinary Medicine, Suez Canal University Approval No. (2022024). A total of twenty-five buffalo calves (*Bubalus bubalis*) of 90-115 days old and 85-135kg were used in the current study. Calves were fed on 50% concentrate feed mixture, 25% rice straw and 25% Egyptian clover to meet the nutritional demands of the growing calves according to NRC (1996). Concentrate mixture was provided twice daily at 8 a.m. and 4 p.m. Clean fresh water was available ad libitum. Thorough clinical examination was performed, and claves were divided according to their dermatological examination into two groups: zinc deficient (G1) group (n=13) that showed abnormal hair coat and skin lesions and apparently healthy (G2) (n=12) with normal hair coat and skin surface. Skin scrapes were obtained from all animals in the two groups followed by KOH 10% and centrifuged at 3000rpm for 5min and examined under microscope for detection of mites.

Blood Samples

One Blood sample was collected from the jugular vein from each animal. One mL of blood was collected in specialized tubes with potassium EDTA for hematological analysis using an automatic cell counter (Methyc 18vet, Orphee, France). The following parameters were measured: Hemoglobin (g/dL), RBCs Count ($\times10^6$/mL), Hematocrit (%), mean corpuscular volume (MCV) (fL), mean corpuscular hemoglobin (MCH) (pg), mean corpuscular hemoglobin concentration (MCHC) (%), Total Leucocytic count (TLC) ($\times10^3$/mL), Neutrophils (%), Lymphocytes (%), Monocytes (%), Eosinophils (%), Basophils (%) and Platelets count ($\times10^6$/mL).

Five mL of blood were collected from each animal in a clean dry tube without EDTA, then to be digested by nitric acid and filtered through a filter paper then tested by flameless atomic absorption spectrophotometer (Aanalyst 100, Perkin Elmer, USA) to determine zinc and copper concentrations.

Behavioral Tests

Behavioral responses of animals in the G1 and G2 groups were assessed using three behavioral tests: the open field test, novel object test and novel human test. These tests are performed in two days in a separate test pen twenty meters away from the home pen with no visual contact to other animals. Food was removed from the test pen during each testing session, but water was supplied in a water trough. Each calf was gently led from the home pen by two familiar workers to the test pen. In the first day, calves were exposed to the open field test (OFT). In the second day, calves were tested by the novel object and novel human tests. Testing order was randomized between days. All testing session were video recorded with a digital camera (Sony hxr-mc2500, Sony, Japan) fixed on a tripod six meters from the test pen. All video recordings were later analyzed by Behavioral Observation Research Interactive Software (BORIS) (Friard and Gamba 2016). The video recordings were analyzed using focal sampling technique (Martin and Bateson 2007).

The Open Field Test (OFT)

The open field test was conducted in a novel arena (13m×10m, L and W, respectively) which had a concrete floor covered with dust similar to the home pen. Each calf was gently guided to the test pen and left there for 10min. The following behavioral activities were scored as durations and later expressed as percentages from the total time of observation; Immobility, Walking, Running, Jumping and Sniffing. The frequency of vocalization was measured as a count. The previous behavioral parameters were chosen according to (de Passillé et al. 1995). Upon completion of the testing session, the subject was then removed from the test pen back to the home pen.

Novel Object and Human Tests

The novel object was a wooden chair placed in the center of the arena. On the next day, each subject was led to enter the arena and the following behavioral activities were scored for 10min.; the latency till first touches the novel object, looking at object, inattentive, touching object and walking. All the behavior data were measured in duration and later expressed as a percentage from the total time except for the latency till first touch the novel object (sec.). Following exposure to the novel object, the object was immediately removed, and an unfamiliar person entered the arena and approached the calf without eye contact. The flight distance (The distance to which the unfamiliar person can approach the calf without causing it to flee) is measured using laser-guided meter (Rangefinder GLM 20, BOSCH, Stuttgart, Germany) by approaching the calf while fixing the laser beam on the body and the first move was recorded as the flight distance.

Statistical Analysis

The SPSS 22.0 software (Armonk, NY: IBM Corp. SPSS, Inc., Chicago, IL, USA) was used for all the comparisons between the two groups. All data set were checked for the assumptions of normality test for each treatment group. Student's t-test was used when the assumption of normality was met, while Mann–Whitney U test was used if the data set was not normally distributed. The level of significance (α) at which the null hypothesis was rejected was 0.05.

RESULTS

The total number of individuals in G1 was 13 individuals, while 12 individuals were found in G2. Age does not significantly (t=0.07, P=0.94) differ between G1 and G2 groups (100.54±1.76 and 100.33±2.29 days, respectively). The average body weights do not significantly (t=-1.62, P=0.12) vary between the two groups although the body weight of the G1 group (106.17±4.58kg) is lower than G2 (116.17±3.83kg).

Blood Copper and Zinc Concentrations

Results from the flameless atomic absorption spectro-photometer for blood copper and zinc concentrations (Fig. 1) showed that there is a significant difference in the mean concentrations of zinc (t=2.41, P=0.03) between G1 and G2 groups, while the mean concentration of copper do not significantly vary between groups (t=1.65, P=0.12). The concentration of zinc is significantly higher in G2 group compared to the G1 group (Fig. 1). Although they did not statistically differ, the blood level of copper is higher in G2 group than G1 (Fig. 1).

Clinical Signs

Clinical examination revealed dermatological lesions and hair coat abnormalities in G1 group which are summarized in Table 1. Dermatological lesions were distributed over neck, dorsum and lateral abdomen (Fig. 2).

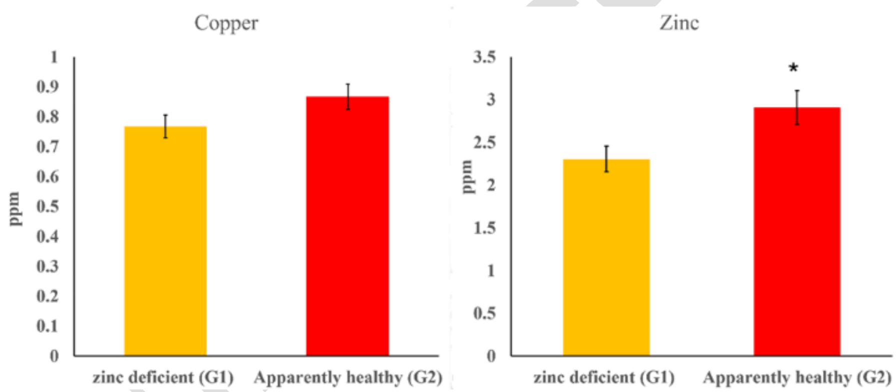

Fig. 1: Blood copper and zinc levels in buffalo calves in zinc deficient (G1) and apparently healthy (G2) groups

Fig.2: Skin lesions and alopecia over dorsum and lateral abdomen in zinc deficient buffalo calves (G1) group.

Table 1: Clinical examination of the skin in buffalo calves in zinc deficient (G1) and apparently healthy (G2) groups

Clinical signs	Zinc deficient (G1)	Apparently healthy (G2)
Skin surface condition	Dry, hard skin Highly keratinized. Fissured skin surface	Soft elastic skin surface
Hair coat	Rough, lusterless hair coat Alopecia on the dorsum and lateral abdominal wall	Glossy and shiny hair coat covering the body
Skin scrape	-ve	-ve
Itching/ licking action	-ve	-ve
Presence of external parasites	-ve	-ve

Table 2: Hematological picture in buffalo calves in zinc deficient (G1) and apparently healthy (G2) groups

Hematological Parameters	Units	Zinc Deficient (G1)	Apparently Healthy (G2)	P value
Hemoglobin	g/dL	12.78±0.60	13.02±0.54	0.77
RBCs Count	$\times10^6$/mL	7.92±0.37	8.18±0.34	0.80
Hematocrit	%	37.70±1.93	37.06±1.81	0.81
MCV	fL	47.70±1.04*	45.18±0.63	0.05
MCH	pg	16.11±0.29	15.87±0.20	0.52
MCHC	%	33.93±0.32*	35.29±0.43	0.02
TLC	$\times10^3$/mL	15.95±1.94	16.98±1.08	0.19
Neutrophils	%	41.22±1.61	38.16±2.25	0.28
Lymphocytes	%	46.57±1.57	48.80±2.23	0.42
Monocytes	%	10.74±0.72	11.77±1.02	0.41
Eosinophils	%	1.48±0.14	1.28±0.13	0.38
Basophils	%	0.00±0.00	0.00±0.00	NS
Platelets count	$\times10^6$/mL	392.72±40.73	366.44±39.10	0.38

Mean corpuscular volume (MCV): Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Total leukocyte count (TLC). *Significant at $P\leq0.05$.

Table 3: Results of novelty tests (Open field, Novel Object and Novel human) in buffalo calves in zinc deficient (G1) and apparently healthy (G2) groups

Behavioral Test	Zinc Deficient (G1)	Apparently Healthy (G2)	P value
A- Open field test			
Immobility (%)	60.94±2.70**	73.44±1.75	0.001
Walking (%)	32.60±2.59**	16.70±1.78	<0.001
Running (%)	3.63±1.14**	0.25±0.17	0.008
Jumping (%)	0.05±0.03	0.00±0.00	0.54
Sniffing (%)	2.77±0.59**	9.61±0.62	<0.001
Vocalization	86.00±7.10**	55.25±7.65	0.007
B- Novel Object Test			
The latency till first touches the novel object (sec.)	290.49±70.21**	46.86±23.57	0.001
Looking at object (%)	2.50±0.48**	7.75±1.45	0.001
Inattentive (%)	62.41±3.76**	76.60±2.19	0.004
Touching object (%)	1.79±0.50**	6.69±1.53	0.008
Walking (%)	29.73±4.29*	15.38±4.93	0.02
C- Novel Human Test			
Flight distance (m)	3.75±0.29*	2.83±0.26	0.03

*Significant at $P\leq0.05$, **Significant at $P\leq0.01$.

Hematological Picture

Comparison of the hematological parameters using Student's t-test between G1 and G2 showed that most of the hematological parameters do not significantly differ between the two groups (except for MCV and MCHC) while the rest of the blood parameters remains insignificantly vary between groups (Table 2). Complete blood picture results revealed a macrocytic hypochromic anemia in zinc deficient group G1 when compared to apparently healthy group G2.

Behavioral Tests

Results of the behavioral activities of the two groups [zinc deficient (G1) and apparently healthy (G2)] are reported in Table 2. In the OFT, the percentages of immobility and sniffing are significantly higher in G2 compared to G1. However, the percentages of walking, running and the number of vocalizations are significantly higher in G1 than G2. The percentage of jumping does not significantly vary between the two groups (Table 3). Also, the results of the novel object test show that the latency till first touch (sec.) and the percentage of walking are both significantly higher in G1 than G2. However, the percentages of looking at object, inattentive and touching object are all higher in G2 than G1 (Table 3). Finally, the flight distance in G1 group is significantly higher than G2 (Table 3).

DISCUSSION

In the current study, buffalo calves in G1 showed abnormal hair coat and skin surface and the laboratory exam revealed zinc deficiency in this group when compared to apparently healthy calves in the G2 group similar to Langenmayer et al. (2018) in his retrospective study in calves and El-Maghraby and Mahmoud (2021) in Egypt, except that the distribution of skin lesion was usually seen as circumscribed areas in the head around the muzzle, periorbital region and on the ventrum. In our study there was a diffused hyper-keratinization on the dorsum, and lateral abdomen which indicates the distribution of dermatological lesions due to zinc deficiency in buffalo calves differ from that in cattle calves. Zinc is an essential trace element that is required for a variety of biological functions in the cell like nucleic acid activity, protein synthesis and carbohydrate metabolism. It is also involved in the synthesis of collagen, and it is primarily more concentrated in the stratum spinosum of skin layers (Michalak et al. 2021). Emri et al. (2015) reported that zinc presence in the keratinocytes facilitates the survival and proliferation of cells. Furthermore, exposure to zinc deficient diet has altered the keratin polypeptides expression which impaired the keratinolytic enzyme function (Ogawa et al 2018), which explains the rough hard dry skin surface in zinc deficient group. Although the hematological values of both groups were not significantly far from the normal CBC references ranges of buffaloes in Egypt according to (Abd Ellah et al. 2013). Zinc deficiency in calves predominantly causes mild microcytic, normochromic anemia Langenmayer et al. (2018), in the current study we found a significant increase in the MCV and significant decrease in the MCHC % in zinc deficient group similar to (El-Maghraby and Mahmoud 2021). Atomic absorption results recorded a non- significant decrease in copper levels in addition to zinc deficiency in this group, which might explain the macrocytic hypochromic anemia found. Copper is involved in the process of cell division and protein synthesis beside it is important for iron absorption by the enterocytes (Huff et al. 2007), meanwhile zinc is a critical element in the structure of metalloenzymes which are responsible for heme synthesis (Kundrapu and Noguez 2018; Samy et al. 2022).

The present results showed that social isolation of zinc-deficient buffalo calves in G1 group triggered clear behavioral changes in the open field test (OFT). The percentages of locomotor activities in G1 group (walking, running and jumping) were found to be significantly higher than G2 group in OFT. Increased locomotor activities and reduced immobility may be a sign of anxiety and stress in mineral-deficient buffalo calves which often reflects an internal tendency to flee the aversive conditions in the OFT and a higher sensitivity threshold to a novel environment (de Passillé et al. 1995). Parallel to our results, studies in laboratory animals showed that zinc-deficient rats displayed higher locomotor activities when evaluated in the OFT than control rats (Takeda et al. 2008). However, some studies reported different results that zinc-deficient rats showed lethargy and a marked decrease in locomotor activities (Gaetke et al. 2002). To explain this discrepancy, it is important to illustrate that the OFT is widely used to assess the negative affective states such as stress and anxiety in a novel arena which demonstrates clearly how the animal responds to the novel environment or the desire to restore social contact to the herd following isolated placement into a novel place (Duncan 2005). Zinc-deficient rats in Gaetke et al. (2002) were assessed in their original home cages which might afford a lower degree of social isolation and risk perception to tested individuals, unlike the current study where buffalo calves were socially isolated and tested in a novel environment. Moreover, the number of vocalizations was found to be significantly higher in G1 compared to G2 group. Strong vocal responses following social isolation might be regarded as an overreaction to novel environmental stimuli and/or an attempt to communicate with the separated herd and usually indicates a heightened anxiety or emotionality responses following social isolation in the OFT (Laurijs et al. 2021).

Curiosity can be defined as the motivation towards gaining novel information and is reflected in approaching and exploring novel objects where there is no instant possibility of reward (Christensen et al. 2021). When animals confronted with a novel object, two contradictory forces may govern their exploratory activities; the first is their constant approach to acquire information and the second is their natural fearfulness toward novel objects which is usually termed "*neophobia*" (Fischer et al. 2016). The current results in both the OFT and novel object test (NOT) revealed that zinc deficient buffalo calves in G1 are more neophobic and fearful than those in G2. This is clearly illustrated by the lower percentage of sniffing in the OFT and the lower percentages of looking at object, inattentive and touching object and the higher latency till approach the novel object in G1 compared to G2. The current results agree with Hagmeyer et al. (2015) who reported that zinc deficiency might be linked with depression, emotional instability, increased anxiety and aggression, irritability and deficits in social behavior besides impaired memory and capacity to learn which may illustrate why individuals in G1 group do not attempt to acquire new information about the novel object. This fear reaction to novelty is also confirmed and generalized to the approach of the unfamiliar person in the novel human test where calves in the G1 group showed higher flight distance when approached with unfamiliar person than G2. Therefore, we may assume that calves deficient in zinc (G1) displayed anxiety and fear-related behavioral responses which are negatively correlated with the exploratory activities (Bak and Malmkvist 2020) than calves in G2. The reason for the behavior of zinc-deficient calves change in the three behavioral tests is unclear. It is assumed that mineral homeostasis plays a significant role for brain function and prevention of brain diseases, and it is likely that brain function and behavior are changed in response to the decrease in zinc concentration in animals suffering zinc deficiency (Takeda et al. 2008). Young rats are fed zinc-deficient food exhibited increased anxiety-like behaviors for 2-week following zinc deprivation (Takeda et al. 2007). Another possible mechanism is zinc deficiency might contribute to activation of the hypothalamic-pituitary adrenal axis (HPA) (Chu et al. 2003) which triggered anxiety and fear responses when animals were confronted with novel places and objects. Further studies should be dedicated to understanding the physiological process behind mineral deficiency in farm animals.

In conclusion, we found that zinc deficiency in weaned calves can affect average body weight and hair coat conditions without severely affecting the hematological parameters. Also, zinc deficiency might induce stress and anxiety which may provoke fear in calves when confronted with novel circumstances.

Author's Contribution

Ibrahim M. Hegab and Walaa I. Mohamaden designed and conducted the study, contributed to data acquisition, contributed to the interpretation of the experimental results and the writing of the manuscript. The authors declare they have no conflict of interests.

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