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Trials for Elevating Adverse Effect of Heat Stress in Buffaloes with Emphasis on Metabolic Status and Fertility

[1]*Hala A.A. Abou-Zeina* [2]*S.G. Hassan,* [2]*H.A. Sabra and* [2]*A.M. Hamam*

[1]Department of Parasitology and Animal Diseases,
Veterinary Research Division, National Research Centre, Giza, Egypt
[2]Department of Animal Reproduction and A.I., Veterinary Research Division,
National Research Centre, Giza, Egypt

Abstract: Heat stress represent a major factor for impairment of fertility in tropical and subtropical countries and need effective applicable strategy for small breeders to overcome the harmful effect of heat stress on health condition and reproductive efficiency of buffalo cows. Twenty mature buffalo-cows, suffering from long post partum anestrum, were divided into 4 equal groups (n=5), treated with Zn (Group 1) ; Zn and Viteslen 15® (sodium selenite and Vit E) (Group 2); supplemented with Zn and Butasyl® (Group 3) or supplemented with Zn and treated with water douches (Group 4). Ovarian activity was detected by rectal palpation and ultrasonography. Some minerals were measured in the soil, ration and in the blood serum. Serum Cortisol, T3 and T4 was measured using ELIZA; serum proteins were fractionated electrophoretically (albumin and α, β andγ globulins) using cellulose acetate membrane technique. Nitric oxide was determined in the serum. Treatment with Zn with or/ without Se+Vit E augment the ovarian activity in 50.0% (2/4) and 20.0% of anestrum buffalo cows, respect., whereas treatment with zinc accompanied with Butasyl or water douching stimulate the ovarian activity in 25.0 and 20.0% of cows, respect. A significant ($p<0.05$) elevation in the levels of serum inorganic P and Zn and drop in the Ca/p ratio following all treatments was recorded. Serum Se was elevated significantly ($P<0.05$) in the group received the antioxidants Zn + Vit E/Se. Antioxidant and anti heat stress exerted significant increasing effects on total serum protein and total globulins, while γ-globulin fraction (Igs) elevated significantly after the treatment with Zn + Vit E/Se. A significant ($P<0.05$) elevation in T3 levels in all trials was recorded. However, the treatment with anti heat stress had a significant ($P<0.05$) decreasing effect on the levels of cortisol. Nitrite, declined significantly ($P<0.05$) in all treated groups, while the depression was more pronounced in the group supplemented with Zn and Vit E/Se. Geochemistry showed that soil was deficient in Zn. Berseem clover offered to animals was found to be deficient in Zn, marginally deficient in Se, while was abundant in Ca leading to adverse effect on P. It is concluded that treatment of anestrum buffalo cows with antioxidants during summer season can improve the fertility of animals and required long term administration. Also, administration of more than one antioxidant had a beneficial effect on health condition and reproductive performance of buffalo more than one antioxidant.

Key words: Buffalo cows · Fertility · Heat stress · Antioxidants · Metabolic status

INTRODUCTION

High ambient temperature or summer heat stress is a major contributing factor to low fertility and immunity among farm animals. It is a world-wide problem, which inflicts heavy economic losses and affects about 60% of the world cattle population. It is clear that reducing heat stress effects on fertility has the potential to results in hundreds of millions of dollars in annual savings to dairy industry. Heat stress, elevating body temperatures, disrupted behaviors and impaired physiological functions [1]. In heat stressed dairy cows there is a reduction in dry matter intake [2-5], which prolongs the period of negative energy balance, which leads to anovulation in dairy cows, especially during the early postpartum period [6,7]. Any worsening of energy balance during summer

Corresponding Author: Hala A.A. Abou-Zeina, Department of Parasitology and Animal Diseases, Veterinary Research Division, National Research Centre, Giza, Egypt

would further decrease fertility in dairy cows at this time. Negative energy balance leads to decreased plasma concentrations of insulin, glucose and IGF-I and increased plasma concentrations of GH and non-esterified fatty acid [7-9]. All of these metabolic hormones can affect reproduction and health condition. Heat stress is also, associated with reduced total antioxidant activity in blood plasma [10] and there is some evidence that the depression in embryo survival following exposure to elevated temperatures involves increased free radical production [11]. However, the short-term administration of the antioxidant vitamin E at the time of AI or at 30 days postpartum had no beneficial effect on pregnancy rate during summer [12], nor did the administration of selenium or β-carotene [13]. In contrast, long-term B-carotene supplements had a beneficial effect on fertility in lactating cows [13,14].

Heat stress is also responsible for large declines in pregnancy rates of dairy cattle during hot months throughout much of the tropical areas, represented in decreased in the fertility of cows inseminated in the late summer months [15- 18], decrease in the conception rate ranged between 20 and 30% compared to the winter season [19-21] and considered the principal cause of extended post partum anestrous period after calving in buffaloes and cattle [22,23]. Oxidative stress might occur when the antioxidants and defense system is overwhelmed by an increased oxidant burden or a reduced antioxidant supply. More recently, the reactive nitrogen species (RNS) have been defined as a subgroup of oxidants deriving from nitric oxide (NO) [24].

Despite its importance, there are few effective strategies for reducing the effects of heat stress on reproduction and health condition. The major strategy providing elaborate housing involving shade, sprinklers, fans, etc., is capital-intensive, not very effective and is of limited use for small and medium-sized dairies as well as for alternative production systems such as grazing dairies. There is thus a need for research into developing alternative approaches. The current study aimed to investigate the effect of antioxidants and anti heat stress on some metabolic status and reproductive performances of Egyptian buffalo cows.

MATERIALS AND METHODS

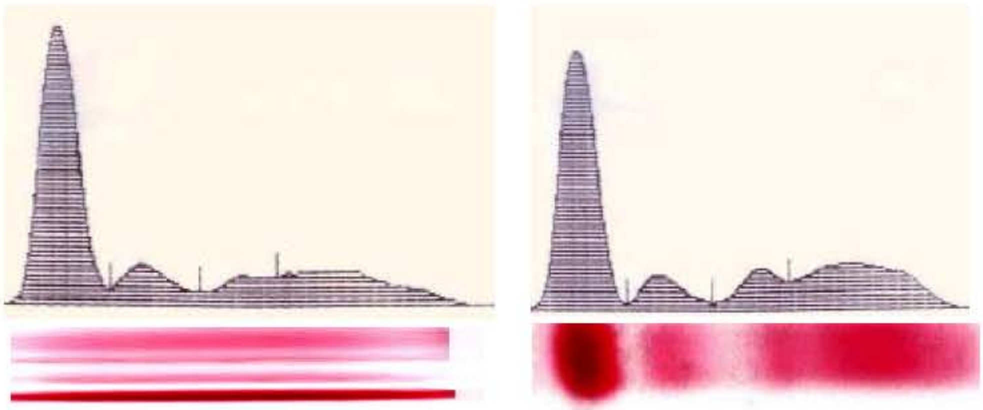
Animal Studies: The present study was carried out at Sids farm, Benisuef Governorate, Animal Reproduction Research Institute, Ministry of Agriculture, Egypt. A total number of 87 apparently healthy female buffaloes aged between 4 to 6 years and identified as lactating, non-lactating and pregnant were rectally examined. Twenty buffaloes were selected depending on their reproductive status where the animals were suffering from prolonged post-partum period and used for the current experiment.
All animals were free from external and internal parasites and were protected against the infections by drenching of albendazole (Pharma, CID) at a dose level of 2 ml/ 20 kg BWT and injection with ivermectin (Ivome Super, Merial) subcutaneously (S/C) at a dose level of 1 ml/50kg BWT before beginning of treatments.

Clinical examination (body temperature, pulse, respiratory rate, physical condition, posture, lymph nodes and others) revealed no incidence of specific symptoms among experimental animals. Also, gynecological examinations were carried out using repeated rectal palpation and ultrasonography (An endorectal linear array 6-8MHz transducer (Scanner 240, PieMedical, the Netherlands) to identify the ovarian activity of buffaloes.

Feeding: In winter, the animals were fed basically on barseem *(Trifolium alexanderinum)*. In summer, each animal given 3kg of concentrate mixture (50% cotton seed cake, 20% rice polish, 15% wheat bran, 7% barley, 5% linseed cake, 2% calcium carbonate and 1% sodium chloride) according to instructions of Ministry of Agriculture. In addition, the animals were given wheat straw (Tiben) *ad libitum* as roughage. Green and concentrated rations offered to the animals were biochemically analyzed for the recognized minerals content [25].

Experimental Procedures: The experiments were designed to investigate the effect of antioxidants and anti heat stress on health condition and some reproductive performances of Egyptian buffalo cows during summer season. The experimental animals were randomly allocated into four equal groups (n=5):

- Group 1, supplemented with Zinc (Zn - methionine) at a dose level of 100 mg once daily/head, for 5 weeks, mixed in limited amount of wheat bran.
- Group 2, received Zn, given at the same way and dose and were injected I/M with Viteslen 15® (Each ml contain, 1.67 mg/ sodium selenite and 150 mg VitE/ml, the Egyptian Co. for Chemicals and pharmaceuticals) at a dose level of 20 ml/head, once/week for 5 weeks.



One dimensional electro gram of a buffalo blood serum before treatment

One dimensional electro gram of a buffalo blood serum after treatment with antioxidants (Zn + Vit E/Se)

Fig. 1: Electrophoretic separation of serum protein fractions using cellulose acetate membrane technique

- Group 3, supplemented with Zn, given at the same way and dose and injected 1/M with butasyl® (butazone anti-inflammatory sod. Saliclate, analgesic and antipyretic) at dose level of 20 ml, head once at the first week, then at dose level of 10 ml, once weekly for 4 weeks.
- Group 4, supplemented with Zn at the same dose and way and treated with water douche at noon for 15 min daily for 5 weeks.

Data of all animals before treatments were used as control.

Sampling and Analytical Techniques: Blood sampling: Blood samples were collected by Jugular vein puncture before initiation of treatments and at the end of experimental period (5 weeks), in plain vaccutainer tubes for serum biochemistry.

Macro and Micro Elements: Serum calcium (Ca) and inorganic phosphorus (P) were determined using kits according to [26] and [27], respectively. Serum Zn levels were measured [28] using flame atomic absorption spectrophotometer (Perkin Elmer, Mod. 3300, USA). Serum Selenium (Se) was measured with Varian Spectra AA 220 Atomic Absorption spectrophotometer equipped with a graphite-furnace tube atomizer (GTA) [29].

Total Serum Protein Levels and Electrophoretic Pattern of Serum Proteins: Total serum protein concentrations were determined directly in a routine manner [30]. The serum proteins were fractionated electrophoretically [31] on cellogel membranes, 2.5 x14 cm cellulose acetate strips (obtained from MALTA S.R.I.-CHEMETRON products, Via Consloe Flaminio, Milano, Italy). The relative densities of stained protein bands (Fig. 1) were determined with the microzone densitometer (B 512 Biotechnical Instrument, Roma, Italy). The absolute values for different protein bands (albumin, α, β and γ- globulins) were calculated according to the values of the total proteins.

Nitric Oxide (NO) Assay: Serum concentration of nitrite, the stable metabolite of NO was measured by a colorimetric method using the Griess reaction [32]. Absorbances of test and standard wells were measured at 570 nm using a Micro titer Plate Reader.

Hormonal Assay

- Determination of triiodothyronine (T_3) and thyroxin (T_4) was carried out [33] using Enzyme Immunoassay test kit (Bio Meriaux, California, USA) and ELISA Reader Stat Fax-2100.
- Serum cortisol was determined by immunometric sequential assay in which anticortisol antibody coated tubes are used [34] using commercial Immulite kits from Diagnostic Product Corporation (DPC, Los Angles, USA).

Soil Samples: Representative samples of soil were collected after soil preparation and before fertilization from the studied area and have been pooled for the geochemistry. Determination of soil pH and electrical conductivity (EC) were carried out in a 1:2.5 soil/water extract [35]. Determination of total calcium carbonate

($CaCo_3$) [36] and organic matter content [37]. Determination of available P [38] and Ca [39]. Estimation of available trace elements; Zn and Se after the wet digestion according to [40] and [25], respectively.

Statistical Analysis: Results were expressed as means±standard error (SE). Differences between means in different groups were tested for significance using a one-way analysis of variance (ANOVA) [41].

RESULTS

Animal Survey: Analyzing the data obtained from the records about buffalo cows in the Sids Farm, Animal Reproduction Research Institute along 5 years revealed that the animals suffering from long post partum period (Table 1) which extends to 446. +126.73 days (M+SD), ranged between 325-950 days. In addition the age at 1st conception was also long (1190.28+720.85 days). The productivity of milk varied greatly between buffalo cows either in the lactation period (176.58+106.61, range 19- days) or in the average milk yield (3.97+1.94, range 1-9.99 kg/day).

Mechanical and Chemical Analysis of Soil: Mechanical analysis of soil samples showed that soil was clay loam (Sand; 14.8%, Silt. 36% and Clay; 49.2%). Geochemistry showed that pH was 8.53, electric conductivity (EC) was 0.21 mmhos/cm, $CaCo_3$ was 2.4% and organic matter was 1.94%.

Macro and micro-elements analysis revealed deficiency of Zn (the ideal content; 1.5-3 ppm) while Ca, P and Se were within the normal levels (Table 2).

Chemical Analysis of Green and Concentrated Rations: Table (2), illustrated the recognized macro and micro elements in Berseem and concentrate rations. Berseem was deficient in Zn and marginally deficient in Se, while Ca and P contents were within the normal levels. The concentrated ration was adequate in macro and micro elements as recommended by NRC (1989).

Gynecological Examination: Gynecological and ultrasonographic examination of a total number of 87 buffalo-cows revealed incidence of anoestrus in 23% of these animals, with inactive ovaries, persistent C.L. and cystic ovaries.

Table 1: Record analysis of some data about the selected buffalo-cows in the herd (farm) during the last 5 years

Item	Age at 1st conception	Post- partum period (Days)	Lactation period (days)	Total Milk yield (kg)/ season	Average milk yield/ day
Average(X̄)	1190.28	446.02	176.58	783.5	3.97
±SD	720.85	126.73	106.61	576.01	1.94
Minimum	498	325	10	19	1
Maximum	2683	950	350	2036	9.99

Table 2: Values of some minerals in the soil, green and concentrated rations in the region of study

Parameters	Soil	Plant (Berseem)	Concentrates	Reference: Soil	Reference: Plant
Ca	260 mg/100gm	1.65%	0.86%	140-260	1-2.5
P	2.10 mg/100gm	0.56%	0.32%	1.2-2.7	0.3-0.5
Zn	1.30 ppm	27.0 ppm	44.50 ppm	1.5-3.0	25-70
Se	0.45 ppm	0.06 ppm	0.31 ppm	0.15-0.85	0.05-0.13

Table 3: Ovarian activity in buffalo-cows treated with antioxidants (Zinc and Vit E /selenium)

		Gynaecological examination (ovarian condition)				
		Before treatment		After treatment		
Treatment	No. of animals	Inactive ovary	Active ovary	Inactive ovary (smooth ovary)	Active ovary (cyclic)	Response to treatments
Zinc methionine	5	5	0	4	(1) Follicle	1/5(20.0%)
Vit E/Se+ zinc methionine	5	4	1 (persistent CL)	2	2 cases suspected early pregnancy. 1 follicle	2/4(50.0%)

Table 4: Ovarian activity before and after treatments for improving adverse condition (Heat Stress)

		Gynecological examination (ovarian condition)				
		Before treatment		After treatment		
Treatment	No. of animals	Inactive ovary (smooth ovary)	Active ovary (cyclic)	Inactive ovary (smooth ovary)	Active ovary (cyclic)	Response to treatments
Anti-inflammatory + zinc methionine	5	4	1 (follicular cyst)	3	2	1/4(25.0%)
Dushing + zinc methionine	5	4	1 (Persistent CL)	4	1	1/5(20.0%)

Table 5: Serum Ca, P, Zn and Se of Buffaloes before and after treatment with antioxidants (Zinc and Vit E / Selenium)

		After treatment		
Parameters	Before treatment N=10	Zinc Methionine N=5	Vit E/ Se + Zn Methionine N=5	P≤
Calcium (mg %)	9.50±0.69[a]	9.66±0.78[a]	9.04±0.57[a]	0.805
Inorganic phosphorus (mg %)	2.68±0.20[b]	4.58±0.23[a]	4.65±0.32[a]	0.0001
Ca / P ratio	3.58±0.22[a]	2.11±0.14[b]	2.00±0.24[b]	0.0001
Zinc (μg %)	66.10±2.62[b]	91.40±6.96[a]	86.80±3.56[a]	0.004
Selenium (μg / L)	27.2 0±4.10[b]	25.40±3.00[b]	58.20±4.70[a]	0.0001

Mean±SE, Means in the same row with different superscripts differently ($p<0.05$)

Table 6: Serum Ca, P, Zn and Se of Buffaloes before and after treatments for improving adverse condition (Heat Stress)

		After treatment		
Parameters	Before treatment N=10	Zn + Douche N=5	Zinc + anti inflammatory N=5	P≤
Calcium (mg %)	9.80±0.68[a]	9.90±0.76[a]	9.06±0.70[a]	0.672
Inorganic Phosphorus (mg %)	2.74±0.25[b]	4.50±0.32[a]	4.48±0.34[a]	0.002
Ca / P ratio	3.78±0.63[a]	2.22±0.18[b]	2.05±0.20[b]	0.016
Zinc (μg %)	70.40±3.47[b]	89.20±6.62[a]	91.20±4.46[a]	0.025
Selenium (μg / L)	28.50±1.40[a]	26.10±1.90[a]	29.00±3.0[a]	0.629

Mean±SE

Means in the same row with different superscripts differently ($p<0.05$)

Table 7: Total Serum protein and electrophoretic patterns of serum proteins of Buffaloes before and after treatment with antioxidants (Zinc and Vit E / Selenium)

		After treatment		
Parameters (g%)	Before treatment N=10	Zinc Methionine N=5	Vit E/ Se + Zn Methionine N=5	P≤
Total Protein	5.03±0.52[b]	7.30±0.32[a]	7.77±0.83[a]	0.036
Albumin	2.67±0.22[b]	3.11±0.21[b]	4.22±0.45[a]	0.032
Total Globulins	2.36±0.30[b]	4.19±0.13[a]	3.55±0.38[a]	0.012
A/G ratio	1.15±0.06[a]	0.74±0.04[b]	1.19±0.03[a]	0.001
α- globulin	0.57±0.076[b]	0.88±0.08[a]	0.86±0.043[a]	0.032
β - globulin	0.44±0.07[b]	1.35±0.19[a]	0.78±0.08[b]	0.006
γ - globulin	1.35±0.17[a]	1.96±0.14[ab]	1.91±0.05[b]	0.044

The effects of antioxidants (Zn and VitE/Se) and anti heat stress (Zn + douche and Zn +anti inflammatory) upon ovarian activity are illustrated in Tables 3 and 4. It was noticed that treatments with the antioxidants (Zn + VitE/Se) stimulate the ovarian activity and induced cyclicity in 50% (2/4) of anestrous buffaloes, whereas, treatment with zinc methionine stimulate the ovarian activity in 20.0% (1/5 of treated anestrous buffaloes. In addition, in the second experiment, only 25.0 and 20.0% of animals treated with Butasyl plus zinc and Duching plus zinc responded to treatment, respectively.

Biochemical and Hormonal Parameters: Macro and micro-elements: Table 5 and 6, revealed that the serum inorganic P and Zn levels increased significantly ($p < 0.05$) in all treated groups compared with levels before treatments. There was significant ($p < 0.05$) decrease in Ca / P ratio in all treated groups. The antioxidants (Zn + VitE/Se) had an increasing significant ($p < 0.05$) effect on the serum concentrations of Se when compared with before treatment levels and the other treated groups.

Table 8: Total Serum protein and electrophoretic patterns of serum proteins of Buffaloes before and after improving adverse condition (Heat Stress)

		After treatment		
Parameters (g %)	Before treatment N=10	Zn + Douche N=5	Zinc +anti inflammatory N=5	P≤
Total Protein	5.23±0.50[b]	7.20±0.55[a]	7.07±0.41[a]	0.053
Albumin	2.88±0.23[a]	3.70±0.32[a]	3.54±0.38[a]	0.234
Total Globulins	2.35±0.28[b]	3.50±0.24[a]	3.53±0.34[a]	0.046
A/G	1.24±0.06[a]	1.05±0.03[a]	1.03±0.16[a]	0.321
α- globulin	0.60±0.06[a]	0.75±0.07[a]	0.77±0.04[a]	0.14
β - globulin	0.42±0.04[a]	0.51±0.04[a]	0.58±0.19[a]	0.064
γ - globulin	1.34±0.21[a]	2.23±0.19[a]	2.18±0.34[a]	0.084

Mean±SE

Means in the same row with different superscripts differently (p<0.05)

Table 9: Serum Thyroid hormones (T3 and T4) and Cortisol levels of Buffaloes before and after treatment with antioxidants (Zinc and Vit E / Selenium)

		After treatment		
Parameters	Before treatment N=10	Zinc Methionine N=5	Vit E/ Se + Zn Methionine N=5	P≤
Tri iodothyronine (T3 - ng/ml)	1.13±0.070[b]	1.48±0.09[a]	1.39±0.02[a]	0.016
Thyroxin (T4 - µg/dl)	12.33±1.10[a]	10.70±0.65[a]	8.21±0.26[b]	0.032
Cortisol (µg/dl)	9.57±0.32[a]	9.14±0.47[a]	8.44±0.50[a]	0.221

Mean±SE

Means in the same row with different superscripts differently (p<0.05)

Table 10: Serum Hormones of Buffaloes before and after treatments for improving adverse condition (Heat Stress)

		After treatment		
Parameters	before treatment N=10	Zn + Douche N=5	Zinc +anti inflammatory N=5	P=≤
Tri iodothyronine (T3 - ng/ml)	1.20±0.05[b]	1.43±0.05[a]	1.54±0.07[a]	0.001
Thyroxin (T4 - µg/dl)	13.07±1.93[a]	8.90±1.29[a]	9.47±0.64[a]	0.16
T3/T4 ratio	0.095±0.016[a]	0.166±0.03[a]	0.16±0.03[a]	0.121
Cortisol (µg/dl)	10.30±1.06[a]	4.50±0.52[b]	3.06±0.79[b]	0.0001

Mean±SE

Means in the same row with different superscripts differently (p<0.05)

Total Serum Proteins and Serum Protein Electrophoresis: Tables 7 and 8 showed that all treatments had significant ($P < 0.05$) increasing effects on total serum proteins and total globulins. Serum protein electrophoresis revealed that the treatment with the antioxidants (Zn + VitE/Se) increased γ-globulin significantly ($p < 0.05$) compared with that treated with Zn alone. There was marked elevation in α, β and γ globulins in the other treated groups.

Hormonal Assay: As shown in tables (9 and10), Serum concentrations of the thyroid hormone T_3 were significantly ($P < 0.05$) increased in all treatments. The administration of Zn+VitE/Se had a significant decreasing effect on T_4 ($P< 0.05$) while the other treatments had a slight effect.

The anti heat stress (Zn + douche) and (Zn + anti inflammatory) had a decreasing significant ($P< 0.05$) effects on cortisol levels, while the antioxidants had slight effects.

Nitric Oxide Assay: serum nitrite levels, the stable metabolite of nitric oxide, in different treated groups are illustrated in Fig 2. All treated groups showed significant ($P < 0.05$) decreases in nitric oxide levels compared with

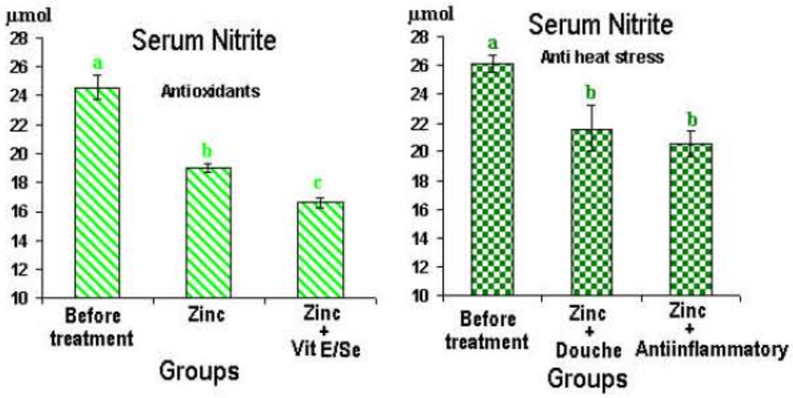


Fig. 2: Levels of nitrite, the stable metabolite of nitric oxide, before and after treatment with antioxidants and anti heat stress in the serum of buffalo-cows

those before treatments. The antioxidants (Zn + VitE/Se) had the most significant effect on nitric oxide levels compared with the other treatments.

DISCUSSION

In heat stressed dairy cows there is a reduction in dry matter intake [2-5], which prolongs the period of negative energy balance, which leads to decreased plasma concentrations of insulin, glucose and IGF-I and increased plasma concentrations of GH and non-esterified fatty acid (7-9). All of these metabolic hormones can affect reproduction and health condition.

With regard to the present work that carried out during summer season; the selected buffalo-cows were suffering from anestrous for long time (Table 1). The analysis of data obtained from the farm in which the experiments were carried out pointed to the delay in conception of buffalo-cows which considered one of the causes of low reproductive efficiency of this species, in addition to the prolong in the post-partum period (446.02±126.73 days, ranged between 325.0 to 950.0 days). In addition to the suppressing role of heat stress on ovarian activity, resulting in long post partum period (anestrum), also there were a deficiency in Zn in the soil and in Berseem (Table 2) and confirmed by analysis of anestrous buffalo serum (Tables 5, 6). Some investigators recorded that Ca, P, Mn, Co, Cu, I, Zn and Se are essential for reproduction and immunity. Dairy cows may suffer from mineral deficiency particularly in the regions were soils are deficient in certain minerals [42], these elements play vital role in the regulation of metabolic and reproductive process [43].

The main diet provided to animals in Egypt during winter season is mainly Berseem clover, which is rich in Ca that exceeded three times the NRC recommendation [44], therefore, excessive Ca interferes with absorption of P and Zn [45]. The soil also suffered from available Zn deficiency [40], which in turn have been reflected on Zn content of Berseem (Table 2) [46]. In previous study [47], zinc deficiency syndromes in buffalo calves have been reported due to its deficiency in the soil and plants. Also Berseem was deficient in Se content (below 0.1 mg/Kg), a value indicating that Se is deficient in animal feed [48]. In dry season, the animals had begun to feed on concentrated ration which was balanced and adequate as NRC recommendations [44], but offered in inadequate amount. This state led to inadequate supplementation with P, Zn and Se, beside the adverse effect of heat stress which lead to impairment of health condition and fertility.

In the present study it is found that the infertile buffalo-cows were suffered from P deficiency, as seen from analysis of animal serum (Tables 5, 6), also, Ca/P ratio was found to be wider before treatment. The obtained results agree with [23, 49, 50], who attributed the ovation dysfunction to the wide range of Ca/P ratio; and contradict with [51, 52] who reported that P deficiency have no influence on ovarian activity in buffaloes. [53], reported that P deficiency decreased fertility in mature cows and is accompanied by anestrus, subestrus or silent heat. Also infertility due to P deficiency can be produced when there is excess of Ca in ration and Pasture that interfere with P metabolism [50].

The obtained results revealed marginal deficiency of Zn in serum of animals, the optimal levels lay between 80-120 µg/dl and the threshold for deficiency is 70 µg /dl [47]. The first effects of marginal Zn deficiency are decreased feed intake, growth, feed efficiency, milk production, resistance to infection and stress and reproductive efficiency [54]. The effect of Zn methionine supplementation was reflected on serum Zn levels of all treated groups, a result that in agreement with [55].

The effect of Se dosing was reflected in serum Se of treated buffaloes that received Vit E/Se, while serum Se levels stabilized at about 25.5 μg/L in other groups lacking Se supplementation. Thus, the buffalo-cows in the present study could be considered marginally deficient in this element, as reported by [56] who defined the marginally deficient serum Se levels to be 20 to 40 μg/L.). Levels of Se and Vit E above the generally accepted requirements enhance the immune response in several species [57-59]. In another study, supplementation of Egyptian buffalo cows with Vit E and Se during the prepartum period, shortened the interval of the first post-partum period to estrus [60].

In the present work supplementation with vitamin E, Se and Zn- methionine(Tables 3,4) for short period improved the fertility of anestrous buffalo-cows and stimulate the ovarian activity in about 50% of treated cows (2/4), whereas, treatment with Zn-methionine alone enhance ovarian activity for about 20.0% (1/5) of treated buffaloes. Many investigators explain the role of Se and Vit E as antioxidants on animal reproduction and their requirements in reproductive tissue [61]. Also, [62] and [63], reported that administration of Vit E and Se improved fertility of buffalo-cows and ewes, respectively. Otherwise, production of free radicals may participate in infertility, because ovarian steroidogenetic tissues are sensitive to free radical damage [64]. It is reported that heat stress is associated with reduced total antioxidant activity in blood plasma [10] and there is some evidence that the depression in embryo survival following exposure to elevated temperatures involves increased free radical production [11]. However, [65] showed no beneficial effect of antioxidants on fertility.

Therefore, administration of antioxidants stimulates the processes of steroidogenesis and evokes the anterior pituitary gland to secrete and release gonadotrsphins and initiation of folliculogenesis in the ovaries. Thus, Vit E/Se could improve uterine health through enhancing neutrophils functions, supports uterine tissue [66] and stimulate ovarian activity [67].

In the present work, the total protein level was markedly declined in the serum of all buffalo cows before treatment (Tables 7, 8). The decline in total protein and its fractions may be attributed to Zn deficiency [45]. In the present study, all the groups received Zn alone, had significantly higher total serum protein and total globulins, whereas, buffaloes received additional antioxidants (Vit E/Se) significantly increased γ-globulin fraction. Data from the present study elucidate that buffaloes supplemented with Zn in combination with Vit E /Se had increased immune globulins (Igs) than did received Zn only. These results agree with numerous studies which support the view that combinations of antioxidants may be more effective than any quantity of single antioxidant [59, 68]; increased the concentrations of natural antioxidants in the blood of sheep [59]. Some authors attributed this effect to increase feed intake due to supplying Zn to cattle which had undergone stress [69]. Otherwise, they hypothesized that Vit E supplementation increased antioxidant recycling and improved synergistic antioxidant effects [70] ; whereas, supplementation with Vit E and Se together have an important synergistic effect on immunity [57-59].

It has been suggested that less than adequate nutrient intake and reduced body condition are major causes of extended post partum an ovulatory intervals, decrease in serum T_3, T_4 and progesterone and suppressed in follicular development in cows [23,71]. Concerning thyroid hormones analysis, the present data demonstrated that all treatments have a positive effect on the T_3, which was significantly elevated after treatment (Tables 9, 10). On the other hand, T_4 showed a numerical depression in all treated groups, except in that supplemented with Zn and Vit E/Se.

Zinc is essential for proper thyroid function. It is involved in T_3 binding to its nuclear receptor [72]. Also, [73] indicated that Zn alone or combined with Se deficiencies resulted in a decrease in thyroid status in rams. Others reported that single and multiple deficiencies of Se, Zn and iodine have distinct effect on thyroid metabolism and structure [74]. Many investigators agree with the present results and confirmed the importance of Se to thyroid hormones metabolism in cattle [75] and in ewes [63]. Also, [75], reported that Se is needed for hepatic conversion of T_4 to 3, 3, 5-triiodothyronine (T_3) and that type I iodo thyronine deiodinase identified as a selenoscysteine containing enzyme, catalyses deiodination of T_4 to biologically active thyroid hormone T_3.

Nitric oxide (NO) is one of the most widespread signaling molecules and participates in virtually every cellular and organ function in the body [76]. Physiological levels of No produced by endothelial cells are essential for vasodilation, regulation of angiogenesis and blood flow in many tissues, including the ovary [76-78]. However, as oxidant and inhibitor of enzymes containing an iron-sulfur center, free radicals and other reactive species cause the oxidation of bio-molecules (e.g., protein, amino acids, lipids and DNA), which leads to cell injury and death [76,79].

Comparing with other treatments, there is marked decrease in the NO activity in the group of animals received Zn and Vit E/Se, which have attributed to the combined anti oxidative effects of the metaloenzymes; superoxide dismutase (Zn) and glutathione peroxidase (Se) which have important role in scavenging harmful free radicals [80,81]. The present finding coordinates with [82].

It is clear that treatment with antioxidant and anti heat stress to elevate the harmful effect of heat stress on health condition and reproductive performance of buffaloes gave a reasonable results but with less extent than expected may be due to short term administration of antioxidant and short period for exposure to water douches.

It is concluded that treatment of buffalo cows with antioxidants during summer season can improve the fertility and immunity of animals and required long term administration. Also, administration of more than one antioxidant had a beneficial effect on health condition and reproductive performance of buffaloes more than one antioxidant.

REFERENCES

1. Mitlohner, F.M., M.I. Galyean and J.J. McGlone, 2002. Shade effects on performance, carcass traits, Physiology and behavior of heat-stressed feedlot heifers. J. Animal Sci., 80: 2043-2050.
2. Hansen, P.J., 1997. Strategies for enhancing reproduction of lactating dairy cows exposed to heat stress. In: Proceedings of the 16th Annual Convention American Embryo Transfer Association, Madison, pp: 62-72.
3. Fuquay, J.W., 1981. Heat stress as it affects animal production. J. Animal Sci., 52: 164-74.
4. Drew, B., 1999. Practical nutrition and management of heifers and high yielding dairy cows for optimal fertility. Cattle Practice, 7: 243-8.
5. Ronchi, B., G. Stradaioli, A. Verini Supplizi, U. Bernabuci, N. Lacetera, P.A. Accorsi, *et al*., 2001. In?uence of heat stress or feed restriction on plasma progesterone, oestradiol-17beta, LH, FSH, prolactin and cortisol in Holstein heifers. Livestock Production Sci., 68: 231-241.
6. Roche, J.F. and M.G. Diskin, 2001. Resumption of reproductive activity in the early post-partum period of cows. In: Diskin M.G., editor. Fertility in the high-producing dairy cow, vol. 26. BSAS Edinburgh: Occasional Publication, pp: 31-42.
7. Butler, W.R., 2001. Nutritional effects on resumption of ovarian cyclicity and conception rate in postpartum dairy cows. In Diskin M.G., editor. Fertility in the high-producing dairy cow, vol. 26. BSAS Edinburgh, Occasional Publication, pp: 133-145.
8. Lucy, M.C., J.D. Savio, L. Badinga, R.L. de la Sota and W.W. Thatcher, 1992. Factors that affect ovarian follicular dynamics in cattle. J. Animal Sci., 70: 3615-3626.
9. Jolly, P.D., S. McDougall, L.A. Fitzpatrick, K.L. Macmillan and K. Entwhitsle, 1995. Physiological effects of under nutrition on postpartum anoestrous in cows. J. Reprodion and Fertility, Suppl, 49: 477-492.
10. Harmon, R.J., M. Lu, D.S. Trammell, B.A. Smith, J.N. Spain and D. Spiers, 1997. In?uence of heat stress and calving on antioxidant activity in bovine blood. J. Dairy Sci., 80(Suppl 1): 264 [abstract].
11. Ealy, A.D., M. Drost, C.M. Barros and P.J. Hansen, 1992. Thermoprotection of preimplantation bovine embryos from heat shock by glutathione and taurine. Cell. Biol. Intl. Reproduction, 16: 125-31.
12. Ealy, A.D., J.L. Howell, V.H. Monterosso, C.F. Are ´chiga and P.J. Hansen, 1994. Changes in thermal sensitivity of preimplantation bovine embryos during development and after treatment with antioxidants. Biol. Reprod, 50(suppl 1): 72.
13. Arechiga, C.F., S. Vazquez-Flores, O. Ortiz, A. Harnandaz-Caron, L.R. Porras, L.R. McDowell and P.J. Hancen, 1998. Effects of injection beta-carotene or vitamin E and selenium infertility of lactating dairy cows. Theriogenol., 50: 65-76.
14. Bonomi, A., A. Quarantelli, A. Sabbioni and P. Superchi, 1994. L'integrazione delle razioni per le bovine da latte con B-carotene in forma rumino-protetta. Effetti sull'ef?cienza produttiva e riproduttiva: contributo sperimentale. Riv. Soc. Ital. Sci. Aliment., 23: 233-49.
15. Ingraham, R.H., D.D. Gillette asnd W.D. Wagner, 1974. Relationship of temperature and humidity to conception rate of Holstein cows in subtropical climate. J. Dairy Sci., 57: 476-81.
16. Ray, D.E., T.J. Halbach and D.V. Armstrong, 1992. Season and lactation number effects on milk production and reproduction in dairy cattle in Arizona. J. Dairy Sci., 75: 2976-83.
17. Thompson, J.A., D.D. Magee, M.A. Tomaszewski, D.L. Wilks and R.H. Fourdraine, 1996. Management of summer infertility in Texas Holstein dairy cattle. Theriogenol., 46: 547-558.

18. Al-Katanani, Y.M., F.F. Paula-Lopes and P.J. Hansen, 2002. Effect of season and exposure to heat stress on oocyte quality of Holstein cows. J. Dairy Sci., 58: 171-182.
19. Cavestany, D., A.B. El-Whishy and R.H. Foot, 1985. Effect of season and high environmental temperature on fertility of Holstein cattle. J. Dairy Sci., 68: 1471-8.
20. Badinga, L., R.J. Collier, W.W. Thatcher and C.J. Wilcox, 1985. Effect of climatic and management factors on conception rate of dairy cattle in subtropical environment. J. Dairy Sci., 68: 78-85.
21. De Rensis, F., P. Marconi, T. Capelli, F. Gatti, F. Facciolongo, S. Franzini *et al*., 2002. Fertility in postpartum dairy cows in winter or summer following estrous synchronization and ?xed time A.I. after the induction of an LH surge with Gonadotropin releasing hormone (GnRH) or human chorionic gonadotropin (hCG). Theriogenol., 58: 1675-87.
22. Hassan, S.G., A.M.A. Abd El-Razek and H. Omima Ezzo, 1991. Studies of some blood constituents in buffaloes during post-partum period. In: Third world Buffobo Congress. Bulgaria. Varna, 13-18: 701-704.
23. Nasr, S.M., H.H. El-Nour, A.L. El-Naggar, E.A. El-Said and M.A. Mahmoud, 2006. Clinicopathological studies on dairy cattle suffering from infertility in New Valley Governorate. Egyptian J. Comparative Pathol. Clinic. Pathol., 19: 1-16.
24. Valko, M., D. Leiffritz, J. Moncol, M.T. Cronin, M. Mazur and J. Telser, 2007. Free radicals and antioxidonts in normal physiological functions and human disease. The Intl. J. Biochem. Cell Biol., 39: 44-84.
25. Association of Official Analytical Chemists (AOAC) (1990). Official Methods of Analysis (13th ed.), Washington, D.C.
26. Gindler, E.M. and J.D. King, 1972. Rapid colorimetric determination of calcium in biologic fluid with Methyl thymol blue. American J. Clinic. Pathol., 58: 376-382.
27. Goodwin, J.F., 1970. Determination of serum phosphorus. Clinic. Chem., 16: 776-780.
28. Fernandez, F.J. and H.L. Kahn, 1971. Clinical Methods for Atomic Absorption Spectroscopy. Clinical. Chemistry News, 1: 3-24.
29. Hoening, M., 1986. A direct determination of selenium in blood and urine samples by platform/Furnace AAS Variance Instruments At Work, No. 61, April.
30. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randal, 1951. Protein measurement with Folin-phenol reagent. J. Biol. Chem., 193: 265-275.
31. Keyser, J.W. and G.L. Watkins, 1972. Estimation of serum proteins by Electrophoresis on cellulose Acetate. Clinic. Chem., 18: 1541-1542.
32. Green, L.C., D.A. Wagner, J. Glogowski, P.L. Shipper, J.S. Wishok and S.R. Tannebaum, 1982. Analysis of nitrate, nitrite and (15 N) nitrate in biological fluids. Anal. Biochem., 126: 131-138.
33. Braveman, L.E., 1996. Evaluation of thyroid status in patients with thyrotoxicoses. Clinic. Chem., 42: 174-181.
34. Burtis, C.A. and E.R. Ashoowd, 1994. Tietz textbook of Clinical Chemistry, 2nd ed. Philadelphia: W.B. Saunders.
35. Chapman, H.D. and P.F. Pratt, 1978. Methods of analysis of soils, plans and water, priced publication 4034. Division of Agricultrual sciences Univ. of California, USA. Preparation of soil sample, P. 5, Vandat Method, pp. 169-170 and pH and EC, pp: 233-236.
36. Alison, L.E. and C.D. Moodle, 1965. Carbonate. In: C.A. Black (ed.), Methods of Soil Analysis. American Society of Agronomy Inc., Madison, Wisc., USA, pp: 1379-1396.
37. Isaac, R.A. and W.C. Johnson, 1984. Methodology of the Analysis of Soil, Plant, Feed, Water and Fertilizer Samples. California Fertilizer Association (CFA), Method S: 18.0 and pp: 80.
38. Olsen, S.R., C.W. Cole, F.S. Watanabe and L.A. Dean, 1958. Estimation of available phosphorus in soil by extraction with sodium bicarbonate, U.S. Department of Agricuture Circular No. 930: 1-19.
39. Jackson, M.L., 1973. Soil chemical Analysis. Prentice-Hall of Indian Limited New Delhi.
40. Lindsay, W.L. and W.A. Norvell, 1978. Development of a DTPA soil tests for zinc, iron manganese and copper. Soil Science. American J., 42: 421-428.
41. Snedecor, G.W. and W.G. Cochran, 1982. Staistical Methods. 8thed., Iowa State University Press, U.S.A.
42. Roderick, S. and M. Hovi, 1999. An assessment of animal health and welfare research priorities in organic livestock production in the UK. A Report to Ministry of Agriculture, Fisheries and Food, UK. pp: 112.
43. Roche, J.F., D. Makey and M.D. Diskin, 2000. Reproductive management of postpartum cows. Animal Reproduc. Sci., 60: 307-312.
44. NRC, 1989. Nutrient Requirements of Domestic Animals, Nutrient Requirements of Dairy Cattle, 6th ed. National Academy of Sciences, National Research Council, Washington, D.C.

45. Underwood, E.J. and N.F. Suttle, 1999. In: The mineral Nutrition of Livestock. 3rd Ed., Midlothian, UK.
46. Nicholas, D.J.D. and A.R. Egan, 1974. Trace Elements in Soil-Plant- Animal Systems. An Academic Press Replica Reprint.
47. Abou-Zeina, H.A.A., 1996. Studies on deficiency of trace elements in livestock. Ph.D. Thesis, (Vet. Med.), Cairo University.
48. Underwood, E.J., 1980. The Mineral Nutrition of Live stock, 159. Commonwealth Agric. Bureaux, Slough,UK.
49. Rgjani, L., M. Brprajapati and S.C. Mrdane, 1995. Haematological and biochemical changes in normal fertile and infertile Surti buffaloes. Indian J. Animal Sci., 65: 536-539.
50. Mostafa, Howayda, E.M., 2002. Clinicopathological findings associated with postpartum ovarian inactivity in buffaloes. M.V. Sci. Fac. Vet. Med. Cairo University.
51. Nosier, M.B., N. Taha and G.A. El Amrawi, 1987. Biochemical investigation in some blood components in fertile and infertile buffalos. Alexandrian J. Vet. Sci., 2: 61-67.
52. El Amrawi, G.A., 1988. An evaluation of selected blood components predictive infertility problems in buffalos, ph. D. Thesis, Alex. Univ.
53. Newar, S., K.K. Baruah, B. Barnah, D. Bhuyan, D. Kalita and A. Baruah, 1999. Effect of mineral constituents in postpartum anestrus Swamp buffalos. Indian Vet. J., 76: 102-104.
54. Spears, J.W., 1991. In “Proc. Grazing Livestock Nutrition Conference” (F.T. McCollum and M.B. Judkins, eds.), P. 138. Agricultural Experiment Station, Oklahoma State University, Stillwater.
55. Wright, C.L. and J.W. Spears, 2001.Effects of zinc source and dietary level on zinc metabolism in Holstein bull calves. J. Animal Sci., 79(suppl.1): 86.
56. Buckley, W., G. Strachon and R. Puls, 1987. Copper and selenium supplementation to calves by means of a soluble glass bolus. Canadian J. Animal Sci., 67: 877.
57. Hassan, H.M., M.H. Nasser, A.A. Ramadan, M.N. El-Sakran and U.M. Mansour, 2001. Effect of vitamin E and selenium as dietary supplements on cellular and humeral immune responses in horses. J. Egyptian Vet. Med. Assoc., 61: 155-164.
58. Kandil, O. M. and H.A.A. Abou-Zeina, 2005. Effect of parenteral vitamin E and selenium supplementation on immune status of dogs vaccinated with subunit and somatic antigens against Taenia hydatigena. J. Egyptian Soc. Parasitol., 35: 537-550.
59. Hamam, A.M. and H.A.A. Abou-Zeina, 2007. Effect of Vitamin E and selenium supplements on the antioxidants on the antioxidant markers and immune status in sheep. J. Biol. Sc., 7: 870-878.
60. Helal, T.S., F.A. Ali, O. Ezzo and M.A. El-Ashry, 1994. Effect of supplementing some vitamins and selenium during the last stage of pregnancy on some reproductive aspects of Egyptian dairy buffalses. J. Agricul. Sci., Mansoura Univ., 19: 4289-4299.
61. Smith, O.B. and O.O. Akinbamijo, 2000. Micro-Nutrients and reproduction in farm animals. Animal Reproduction Sci., 60-61: 544-560.
62. Helal, F.J.,W.M. Ahmed, M.M. El Ashry, H.M. El Sayed and A.M. El-Hanafy, 1998. Effect of supplementing selenium and vitamin E on some productive and reproductive traits in lactating buffalo cows. 2-Reproductive performance. J. Agricul. Sci., Mansoura Univ., 23: 2125-2137.
63. Abou-Zeina, H.A.A. and A.M. Hamam, 2002. Effects of parenteral administration of vitamin E and / or selenium on health and reproductive function of ewes fed on marginal deficient diets. Egypt. J. Basic and Appl. Physiol., 1: 205-223.
64. Carlson, J.C., M. Wux and Sawoda, 1993. Oxygen radicals and the control of ovarian corpus luteum function. Free Radical of Biological Medicine, 14: 79-84.
65. Stowe, H.D., T.W. Thomas, T. Johnson, J.V. Marteniuk, D.A. Morrow and D. Ullrey, 1988. Response of dairy cattle to long terms and short term supplementation with oral selenium and vitamin E., J. Dairy Sci., 71: 1830-1839.
66. Politis, I., N. Hindiroglou, J.H. White, J.A. Gilmore, S.N. Williams, H. Scherf and M.Frigg, 1996. Effects of vitamin E on mammary and blood leucocyes function, with emphasis on chemotaxis in periparturient dairy cows. American J. Vet. Res., 57: 463-471.
67. Meshreky, S.Z. and G.H. Metry, 2000. Effect of vitamin E and/or selenium injection on: I. Reproductive performance of New Zealand and White and Baladi Black doe rabbits under climate conditions of middle Egypt. In the proceeding of 3rd All Africa conference on Animal Agriculture and 11th conference of the Egyptian Society of animal production, 6-9 Nov., Alexandria, Egypt.
68. Young, A.J. and G.M. Lowe, 2001. Mini review: Antioxidant and pro oxidant properties of carotenoids. Archive Biochem., 385: 20-27.
69. Spears, J.W. and E.B. Kegley, 1991. Effect of zinc and manganese methionine on performance of beef cows and claves. J. Animal Sci., 69 (Suppl. 1): 59.

70. Kirschvink, N. de, M.B. and P. Lekeux, 2007. Review: The oxidant / antioxidant equilibrium in horses. The Vet. J., doi: 10.1016/J. TvJl. 2007.07.033.
71. Bossis, I., R.P. Wettemann, S.D. Welty, J.A. Vizcarra, L.J. Spicer and M.G. Disken, 1999. Nutritionally induced an ovulation in beef heifers: ovarian and endocrine function preceding cessation of ovulation. J. Animal Sci., 77: 1536-1546.
72. Liu, N., L. Pingsheng, X. Qing Z. Li, Z. Zhiying, W. Zhenghua, L. Yanfen, F. WeiJing and Z. Lianzhen, 2001. Elements in erythrocytes of Population. Trace Element Res., 84: 37-43.
73. El-Tohamy, M.M., 2002. Effect of single and combined zinc-selenium deficiencies in rams. XXII World Buiatrics Congress, Hannover, Germany.
74. Ruz, M., 1995. Trace element intake and nutrition in Latin America. In: Nutrition in a Sustainable Environment (Walhlqivist, M.L., *et al.*) pp: 296-300. Smith - Golden and Co., London, UK.
75. Arthur, J.R., F. Nicol and G.J. Beckett, 1993. Selenium deficiency, thyroid hormone metabolism and thyroid hormone deiodinases. American J. Clinic. Nutr., 57 (suppl.): 2365-2395.
76. Ignarro, L.J., G. Cirino, A. Casini and C. Napoli, 1999. Nitric oxide as a signaling molecule in the vascular system: an overview. J. Cardiovascular Pharmacol., 34: 879.
77. Cooke, J.P., 2003. NO and angiogenesis. Atherosclerosis Supplements, 4: 53-60.
78. Duda, D.G., D. Fukumura and R.K. Jain, 2004. Role of e NOS in neovascularization: NO for endothelial progenitor cells. Trends in Molecular Medicine, 10: 143-145.
79. Freidovich, I., 1999. Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? Annual NY Academy Science, 893: 13.
80. Rotruck, J.T., A.L. Pope, H.E. Ganther, A.B. Swanson, D.G. Hafeman and W.G. Hoekstra, 1973. Selenium: Biochemical component of glutathione peroxidase. Sci., 179: 585-590.
81. Teoh, M.L.T., P.J. Walasek and D.H. Evans, 2003. Leporipoxvirus Cu, Zn-super oxide dismutase (SOD) homologs are catalytically inert decoy proteins that bind copper chaperone for SOD. J. Biol. Chem., 35: 33175-33184.
82. Rajarman, V., B.J. Nonnecke, S.T. Franklin, D.C. Hammell and R.L. Horst, 1998. Effect of Vitamins A and E on Nitric oxide production by blood mononuclear leukocytes from neonatal calves fed milk replacer. J. Dairy Sci., 81: 3278-3285.

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