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Investigations on Delayed Puberty in Egyptian Buffalo-Heifers with Emphasis on Clinicopathological Changes and Treatment Using GnRH (Receptal®)

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Abstract: The delay in puberty and consequently delayed conception is one of the most important causes of low reproductive efficiency in buffaloes. The present study was designed to throw light on this phenomenon and to make a treatment trial to improve the productive performance of Egyptian buffaloes. This study was conducted during the breeding season of buffaloes (September - March) on 200 buffalo-heifers reared at Lower Egypt and kept in smallholder farm. Animals were gynecologically examined and blood samples were collected to investigate some clinicopathological changes associated with this phenomenon. Also, fecal samples and vaginal swabs were collected from all heifers. Heifers were divided into two groups; the 1st group included 70 normal pubertal animals, kept as the control group and the 2nd group included 130 animals represented the delayed pubertal buffalo-heifers.37 delayed pubertal heifers were intramuscularly (im) injected with 200µg of GnRH (Receptal®) and response was recorded. Results indicated that the most recorded haematological changes associated with this phenomenon were macrocytic hypochromic anemia, changed red cell parameters and decreased total leucocytic count (TLC), neutrophils and lymphocytes with oesinophilia. Decreased values of serum progesterone (P_4), glucose, cholesterol, total proteins, albumin, globulins, ceruloplasmin (Cp), calcium, inorganic phosphorus (P), zinc (Zn) and copper (Cu) were obvious in delayed pubertal heifers with marked changes in oxidant/antioxidant parameters. Prevalence of parasitic infestation was higher in delayed pubertal than normal pubertal buffalo-heifers. Also, vaginal bacterial isolates were higher in delayed pubertal than normal pubertal buffalo-heifers with low incidence of *lactobacilli spp.* isolation. 75.68% of treated heifers revealed estrous activity within 20 - 30 days post GnRH treatment with obvious ovarian activity. In conclusion, delayed puberty is a common phenomenon in buffalo heifers and is associated with obvious clinicopathological changes, disrupted oxidative status and low serum progesterone level. GnRH efficiently treated this condition in 75% of the cases. Proper nutrition and biosecurity are the main keys to avoid the occurrence of delayed puberty in buffaloes.

Key words: Buffaloes · Heifers · Puberty · Clinicopathological changes · Biosecurity · Oxidative status-GnRH

INTRODUCTION

Buffaloes represent an integral part of the agricultural economy in Egypt and some other developing countries. This species is mostly reared in smallholder farms under harsh socioeconomic conditions and showed low productive and reproductive performances. Seasonality of breeding and reproductive problems; mainly delayed puberty, ovarian inactivity and endometritis are the main obstacles for improving this species and caused great economic losses [1, 2].

In buffaloes, many factors including breed, season of birth, climate, feeding, growth rate, biosecurity of the animals and bull exposure affect age and weight at the onset of puberty leading to a large differentiation for reproduction performances in buffalo-heifers [3]. Normal

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physiological processes including attainment of puberty required application of biosecurity which is an attempt to keep infectious agents (e.g., bacteria, virus and parasites) away from a herd [4].

Gonadortopin releasing hormone (GnRH) is available hormonal preparation in the market and has been used to improve reproduction in farm animals, particularly in bovines whereas, treated heifer calves with exogenous GnRH from 4 to 8 weeks of age and revealed subsequent increased in BW gain, mean LH concentrations and LH pulse frequency. The GnRH treatment also reduced age at puberty [5].

As there are limited systematic investigations on delayed puberty phenomenon in buffaloes, the current study was planed to carrying out some investigations on delayed puberty phenomenon under the prevailing Egyptian conditions with emphasis on some associated clinicopathological analyses and to carry out a treatment trial to improve the productivity of Egyptian buffaloes via shortening the non-productive period in the lifetime of the animal.

MATERIALS AND METHODS

The current work was carried out on 200 buffalo-heifers reared in small holder farms at Lower Egypt during the period from September, 2006 to March, 2008. Heifers were divided into two groups; the 1st group included 70 normal pubertal animals, kept as the control group (15-17 months) according to Youssef *et al.* [6] and the 2nd group included 130 animals represented the delayed pubertal buffalo-heifers their ages above 17 months (18-36 months). Age was determined depending on dentations values described by Fraser [7], case history was recorded and animal was examined for general body condition status and the presence of signs of deficiency. Also, gynecological examination was carried out as outlined by Youngquist [8] aided by ultrasonography using ultra sound apparatus (Pia Medical Falcs e`Saote, the Netherlands) with an endorectal linear array 6-8 MHz transducer according to Terzano [9].

Sampling: Blood samples were collected either on EDTA for performing complete blood picture and determination of glutathione reduced (GSH) or without any anticoagulant for separation of serum (x1500g for 10 minutes, 4°C) then stored at -20°C until used for biochemical analysis.

A sufficient quantity of feces was collected and placed in a suitable air-tight plastic bag for fecal examination by using saturated sodium chloride solution (flotation sedimentation technique) according to Soulsby [10].

Bacteriological swabs were collected from anterior vagina using the under possible hygienic conditions and inserted into nutrient and M.R.S broth according to Youngquist [8].

Laboratory Analysis: Complete blood picture was done according to standard techniques described by Feildman *et al.* [11].

Serum progesterone level was determined by ELISA kits (DIMA, Germany) using the micro- well method. The kit had a sensitivity of 2.0 pg/ml with the inter- and intra-run precision coefficient of variations of 2.9 and 4.85, respectively [12]. Other serum biochemical values including Malondialdhyde (MDA), nitric oxide (NO), catalase (CAT), superoxide dismutase (SOD), vitamine C (ASCA), glutathione-reduced (GSH), total antioxidant activity (TAA), ceruloplasmin (Cp), progesterone level (P_4), glucose, total protein, albumin, cholesterol, calcium and inorganic phosphorus (P) were carried out using commercial chemical kits (Biodiagnostic, Egypt). Serum zinc (Zn) and copper (Cu) values were determined using atomic absorption spectrophotometery as described by Fernandez and Kahn [13].

Treatment Trial: A field treatment trial was carried out whereas; 37delayed heifers were intramuscularly injected with the recommended dose (200 µg) of GnRH (Receptal®, Intervet, the Netherlands) and the heifers were followed up for the occurrence of heat signs and the genital organs were weekly examined and response to treatment was recorded.

Statistical Analysis: Data were computed and statistically analyzed using Student "t" test [14].

RESULTS

Delayed pubertal heifers showed poor body condition with no signs of estrus during the breeding season of buffaloes with very small genital organs and had no physiological structures in their ovaries as shown by ultrasonographic examination. Serum progesterone level was not detectable in delayed pubertal heifers (<0.002 ng/ml), while this level was 0.379±0.03 and 2.00±0.23 ng/ml in normal cyclic heifers during the follicular and luteal phases of the estrous cycle, respectively.

Table 1: Hematological changes associated with delayed puberty in buffalo heifers (Mean ± SE)

Groups Parameters	Normal pubertal heifers (N=24)	Delayed pubertal heifers (N=30)
-RBCs ($\times10^6$/µl)	5.96±0.02	5.18±0.11***
-Hb (g/dl)	11.24±0.14	10.17±0.14***
-PCV (%)	31.04±0.23	28.07±0.33***
-MCV (fl)	52.10±0.53	54.61±0.86*
-MCH (pg)	19.71±0.19	18.87±0.29*
-MCHC (%)	36.18±0.19	36.01±0.23
-WBCs ($\times10^3$/µl)	7.5±0.11	6.3±0.15***
-Neutrophils ($\times10^3$/µl) (%)	2.81±0.11	2.08±0.05***
	(36.04±0.18)	(33.10±0.38)***
-Lymphocytes ($\times10^3$/µl) (%)	4.05±0.06	3.23±0.08***
	(54.04±0.30)	(51.20±0.26)***
-Monocytes ($\times10^3$/µl) (%)	0.48±0.02	0.43±0.02
	(6.42±0.18)	(6.87±0.18)
-Eosinophils ($\times10^3$/µl) (%)	0.26±0.01	0.56±0.02***
	(3.46±0.16)	(8.83±0.29)***
-Basophils ($\times10^3$/µl) (%)	0.003±0.003	0.000
	(0.04±0.04)	(0.00)

* P<0.05. *** P<0.001

Table 2: Some serum biochemical changes associated with delayed puberty in buffalo-heifers (Mean ± SE)

Groups Parameters	Normal pubertal heifers (N=10)	Delayed pubertal heifers (N=10)
- Blood glucose (mg/dl)	65.59±1.23	51.61±2.18***
- Cholesterol (mg/dl)	152.26±4.79	124.07±2.36***
- Total proteins (g/dl)	5.33±0.09	4.78±0.11***
- Albumin (g/dl)	3.67±0.05	3.41±0.03***
- Globulins (g/dl)	1.67±0.09	1.19±0.09**
- A/G ratio	2.26±0.13	2.56±0.07
- Ceruloplasmin (mg/dl)	27.78±0.80	11.54±0.27***

** P<0.01. *** P<0.001

Table 3: Some mineral and trace element values associated with delayed puberty in buffalo-heifers (Mean ± SE)

Groups Parameters	Normal pubertal heifers (N=10)	Delayed pubertal heifers (N=10)
- Ca (mg/dl)	10.23±0.21	9.07±0.10***
- P (mg/dl)	4.85±0.30	3.65±0.08**
- Ca/P ratio	2.15±0.08	2.49±0.03**
- Zn (µg/dl)	158.4±5.49	119.0±1.99***
- Cu (µg/dl)	185.7±2.60	93.3±3.12***

** P<0.01. *** P<0.001

Table 4: Changes in Oxidant/antioxidant markers in serum of buffalo-heifers associated with delayed puberty (Mean ± SE)

Groups Parameters	Normal pubertal heifers (N=15)	Delayed pubertal heifers (N=15)
- Malondialdhyde (MDA, mmol/L)	2.09±0.12	3.68±0.12***
- Nitric oxide (NO, µmol/L)	24.2±0.70	31.52±0.85***
- Catalase (CAT, U/ml)	2.65±0.07	0.87±0.03***
- Superoxide dismutase (SOD,U/ml)	340.07±2.79	305.91±2.72***
- Ascorbic acid (ASCA, µgm/L)	133.26±1.07	133.13±1.01
- Glutathione-reduced (GSH, mmol/L)	7.4±0.18	3.76±0.11***
- Total antioxidant activity (TAA, mmol/L)	1.46±0.02	0.44±0.02***

*** P<0.001

Table 1 indicates that the delayed pubertal heifers revealed decreased (P<0.001) erythrocytic parameters (RBCs count, packed cell volume and hemoglobin contents), with clear macrocytic hypochromic anemia. In the same time, these animals revealed decreased (P<0.001) TLC, neutrophils and lymphocyte with eosinophilia.

Table 2 shows that delayed pubertal heifers revealed decreased blood glucose, cholesterol, total proteins, albumin and ceruloplasmin (P< 0.001) as well as globulins (P<0.01) concentrations as compared with normal pubertal heifers.

Table 3 reveals decreased serum calcium (P<0.001) and inorganic phosphorous (P<0.01) concentrations with wide Ca/P ratio (P<0.01) and zinc and copper (P<0.001) concentrations in delayed heifers compared with the normal pubertal heifers.

Table 5: Prevalence of parasitic infestation in buffalo-heifers with normal and delayed puberty

Groups Parasites	Normal pubertal heifers (N=70)	Delayed pubertal heifers (N=130)
Ascaris	15 (50.0%)	30 (47.62$ %)
Fasciola	0 (0.0%)	9 (14.29%)
Coccidia	15 (50.0%)	24 (38.10%)
Total incidence of parasitic infestation	30 (42.86%)	63 (48.46%)

Table 6: Bacterial isolates from vagina of buffalo-heifers with normal and delayed puberty

Groups Parameters	Normal pubertal heifers (N=20)	Delayed pubertal heifers (N=20)
Bacterial isolates:-		
- Gram positive bacteria: -		
- *Corynebacterium spp.*	0	4
- *S. epidermidis*	0	2
- *Micrococcus spp.*	2	12
- *E. faecalis*	1	4
- Gram negative bacteria: -		
- *Y. enterocolitica*	2	8
- *E. coli*	4	16
- *C. diversus*	0	12
- *K. oxytoca*	0	10
- *Pasturella spp.*	0	2
- *Proteus spp.*	0	2
- Incidence of bacterial isolates	5 (25%)	18 (90%)

*** P<0.001

Table 7: Isolation of *Lactobacillus* strains from the vagina of the buffalo-heifers with normal and delayed puberty

Groups *Lactobacillus strains*	Normal pubertal heifers (N=20)	Delayed pubertal heifers (N=20)
- *L. casi rhamnosus*	9 (45%)	1 (5%)
- *L. yamanashiensis*	1 (5%)	1 (5%)
- Incidence of *Lactobacillus spp.*	10 (50%)	2 (10%)

*** P<0.001

Table 8: Treatment of same delayed pubertal buffalo-heifers with synthetic GnRH ((Receptal®)

Parameters	(Receptal®).
Number of delayed heifers	37
Number of responded	28 (75.68%)

Table 4 indicates that delayed pubertal heifers have high MDA (P<0.001) and NO with low CAT, SOD, GSH and TAA(P<0.001) values as compared to normal pubertal animals.

Table 5 shows that the most predominate parasites in the delayed pubertal heifers were *Ascaris, Coccidia* and *Fasciola*, but in normal pubertal heifers the most predominant parasites were *Ascaris* and *Coccidia*. However, the incidence of parasitic infestation was higher in the delayed heifers compared than in normal pubertal heifers (48.46 *Vs.* 42.86 %).

Table 6 reveals that the incidence of vaginal bacterial isolates was higher in delayed pubertal heifers (90%) than in the normal pubertal buffalo-heifers (25%).

Table 7 illustrates that the incidence of vaginal *Lactobacillus spp*. was lower in delayed pubertal heifers than normal pubertal buffalo-heifers. The most predominant strains isolated from the delayed pubertal buffalo-heifers were *L. casi rhamnosus* and *L. yamanashiensis,* but *L. casi rhamnosus* and *L. yamanashiensis* were isolated from the normal pubertal heifers.

Table 8 indicates that Receptal® induced signs of estrous activity in 75.68% of the treated heifers within 20 - 30 days post injections. Rectal palpation aided by ultrasonography confirmed the occurrence of ovarian activity indicated by the presence of mature Graafian follicles and/or Corpora lutea.

DISCUSSION

In Egypt, buffaloes have a unique role in livestock production and the agricultural economy whereas, it produces about 65% of total consumable meat and milk

[15, 16]. However, the productivity of these animals is limited by poor reproductive efficiency, seasonality, reproductive disorders as long post-partum anoestrus and it is further hampered by several external environmental factors mainly; nutrition and parasitism [17].

From the clinicopathological point of view, delayed puberty heifers revealed macrocytic hypochromic anemia with significant decrease in erythrocytic parameters (RBCs, PCVand Hb); these findings coincided with those given by Ahmed *et al.* [15] whereas, they found that blood picture of non cyclic buffalo-heifers revealed significant decreased of erythrocytic count, Hb content, PCV and MCHC together with increased MCV. The causes of decreasing erythrocytic paramerters may be attributed to the direct effect of under nutrition or the indirect effect associated with parasitic infestation [18] as well as Cu deficiency. It was found that iron transport within the body is adversely affected and tended to accumulate in many tissues following Cu deficiency [19]. Moreover, Hb content in Cu deficient animals was significantly low [20]. Cu is essential for erythrocyte production [21]. Cardoso *et al.* [22] reported that hypochromic anaemia is related to Cu deficiency.

Delayed pubertal heifers showed significant decrease in TLC, neutrophils and lymphocyte, these findings might be due to absence of estrogen which is responsible for normal cellular and humeral immune response in heifers [23]. Migration of leukocytes to be infiltrated in tissue of genital tracts may be another cause, especially in cases associated with bacterial infection. Also, it was reported that unsuitable agroclimatic conditions are associated with low leucocytic count [24]. On the other hand, the significant eosinophilia in the current delayed heifers was a normal consequence to parasitic infestation as demonstrated by Ahmed and Hassan [25].

In the present work, significant low values of calcium and inorganic-phosphorous, total proteins, albumin, globulins, glucose and cholesterol concentrations were assayed in the serum of delayed pubertal heifers as compared with normal animals. Similar findings were recoded by Abdoon *et al.* [26] who related these results to ration formulation, management and agroclimatic conditions. Also, it was found that the parasitic infestation has a detrimental effect on serum total proteins, albumin, globulin, calcium and inorganic phosphorous concentrations [27].

Serum Cp concentration was significantly lower in delayed pubertal as compared with normal pubertal heifers. Sharma *et al.* [20] observed a significant decreased of serum Cp in the Cu deficient animals. They advocated the use of Cp: Cu ratios in the diagnosis of induced Cu disorders in ruminants. Although serum Cp decreased by Cu deficiency, the relative concentration increased during infection [28]. Cp also promoted the incorporation of iron into the storage protein, ferritin [29].

The present study clarified low concentrations of Zn and Cu in delayed compared with the normal pubertal heifers. These findings agreed with those of Saxena *et al.* [30] who found a correlation between serum Cu and Zn concentrations and age at puberty in crossbred heifers, but Small *et al.* [31] did not find serum Zn, Cu, or Mg concentrations to be related to first-service conception rates in cattle. However, in heifers loss of appetite and reduced fertility are seen even in Cu deficiency, along with interference in the release of LH. It was suggested that the cause is the inhibitory effect of estradiol in the pituitary gland due to sex hormonal disturbances [32]. However, Cu absorption in ruminants is low (>1.0-10.0%) relative to values reported in non-ruminants [33]. The percentage of dietary Zn that is absorbed decreased as dietary Zn increased in ruminants. Zn requirements of ruminants appear to be affected by dietary factors based on the variable animal responses that were observed after Zn supplementation. However, dietary factors that affect Zn bioavailability in ruminants are not clearly defined [34]. Moreover, it was found that Zn deficiency in the female lead to problems such as impaired synthesis/secretion of follicular stimulating hormone FSH and LH, abnormal ovarian development, disruption of the estrous cycle, frequent abortion, a prolonged gestation period, teratogenicity, stillbirths, difficulty in parturition, pre-eclampsia, toxemia and low birth weights of infants [35]. Ahmed *et al.* [36] noticed that heifers suffered from Zn and Cu deficiencies show delayed puberty, stunted growth and infertility. In the same time, it was reported that supplementation of deficient dairy cows with Zn improve reproductive performance via shorting interval (30 days) from calving to resumption of estrus as compared to control (69 days), [37].

The present study highlighted the association between oxidative stress and depleted antioxidant system on one hand and the occurrence of delayed puberty in buffalo-heifers on the other hand whereas, the concentrations of some oxidant/antioxidant markers revealed a significant increase of MDA and NO with a significant decrease of CAT, SOD, GSH and TAA in the delayed heifers compared with the normal pubertal heifers. Similar results reported by Ahmed *et al.* [14, 15], whereas, they found a tight relationship between oxidative stress and ovarian inactivity in buffalo-heifers. This implies that the delayed pubertal heifers were under stress condition.

Oxidative stress has been implicated as a major initiator of tissue damage and could affect enzymatic activity, signal transcription and gene expression, especially apoptotic gene [38]. Moreover, it was reported that oxidative stress plays a number of significant roles in female reproductive biology; mainly it influences ovarian function by affecting the growth of Graafian follicles and oocyte maturation [39]. Shimamura *et al.* [40] reported that ROS have anti-gonadotrophic and anti steroidgenic actions in rat luteal cells.

The present work showed that the prevalence of parasitic infection is higher in the delayed heifers as compared with the normal pubertal heifers (48.46 *Vs.* 42.86 %,). These results coincided with the finding of other studies which found that parasitized animals showed a significant delay in the onset of puberty, depressed appetite and consequently decreased weight gain, also has a negative impact on the reproductive efficacy of dairy heifers via alteration the serum concentration of estrogen, which in turn results in an abnormally low concentration of P_4 [41]. Mejia *et al.* [42] found that the treatment with Ivermectin totally eliminated the presence of nematode eggs in feces and resulted in earlier onset of puberty. Subclinical parasitic infestation affected nutrient utilization and altered animal metabolism so anthelmintic administration decreases both age and weight of an animal at puberty [18].

Vaginal bacterial infections are important because they not only disrupt function of the uterus, but also the ovary and the overarching higher control centers in the hypothalamus and pituitary [43]. The current work indicated that the incidence of vaginal bacterial isolates was higher in delayed than normal pubertal buffalo-heifers. Abd El-Moez [44] found that the rate of isolation of bacteria from the genital tract of female buffaloes is the least in heifer approaching puberty, whereas the estrogen level increased which has a bactericidal effect. Also, immune/inflammatory mediators have a disruptive effects included impaired follicular development and a delayed or blocked preovulatory LH surge as demonstrated by Suzuki *et al.* [45].

Lactobacillus spp. is one of probiotic bacteria which, beneficially affect the host by improving its microbial balance health restoration and maintenance of the genital tract [46], including eliminating or reducing microorganism that are carried by the host and that harmful to humans [47] via the production of lactic acid which acidifies the vagina, hydrogen peroxide and bacteriocin [48].

In the current investigations, treatment of some delayed pubertal heifers with GnRH induced clear signs of estrus in 75.68 of the treated heifers within 20 - 30 days post injection and the ovarian activity was confirmed by ultrasonography. In this respect, it was recorded that most buffaloes released LH surge in response to exogenous GnRH imjection [49]. GnRH cause rapid secretion of LH and FSH from the pituitary with subsequent elevations of these hormones in the peripheral circulation and consequently ovarian activity [50].

In conclusion, delayed puberty is a common phenomenon in buffalo heifers and is associated with obvious clinicopathological changes; including macrocytic hypochromic anemia, leukocytopenia with oesinophilia, hypoproteinemia and disrupted oxidative status. Progesterone level is the mirror for the ovarian activity in buffalo heifers. GnRH efficiently treats 75% of the effected animals. Sufficient nutrition and proper biosecurity are the main keys for shortening the non-productive period of buffalo-heifers.

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