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Prospects of GnRH-loaded on chitosan nanoparticles combined with exogenous progesterone supplementation for treatment of true anoestrus in multiparous Egyptian buffaloes during summer months

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ABSTRACT

This study was designed in order to clarify the follicular dynamics, steroid hormones concentrations, and conception rate in true anestrus buffaloes during the summer months. Before beginning, true anestrus was diagnosed via trans-rectal ultrasonography on day 85 and day 95 postpartum by SonoScape scanning. 50 true anestrus multiparous lactating Egyptian buffalo cows with average ages 5-8 years; weighted 400- 450kg, parity 3±0.2 and daily milk production of 10.4±0.6 kg were chosen. Two protocols were applied (GPP group: GnRH-PRID-synch protocol, n=20 and GCP group: GnRH-loaded chitosan-PRID-synch protocol, n=20) and compared with control group, n=10. In day 0 for GPP group, PRID was inserted intravaginally together with 2 ml Gonavet Veyx® (commercial GnRH), then it removed at d7 and buffaloes received 2 ml Estrumate. On day 9, animals received 2 ml Gonavet Veyx® intramuscular and then were artificially inseminated at fixed time 16-20 hr later. The same protocol was performed on GCP group, but Gonadotropins were modified by loading GnRH on chitosan nanoparticles so, buffaloes were recived 1 ml of modified Gonavet Veyx® at d0 and d9. Control group buffaloes received 10 ml intramuscular saline (NaCl 0.9%) on Days 0, 7, and 9, respectively. The serum concentration of (E2 and P4) was estimated. Ultrasonography was used for diagnosis of follicular dynamics. Estrus induction and pregnancy rates were calculated. GCP group had the highest values of E2, P4 and CL size. In addition, improved estrus induction and pregnancy rate in GCP group were noticed compared to other groups but without significant differences.

Introduction

The continuity of buffalo's production essentially depends on the efficiency of its reproduction which was impaired by late maturity age, low estrus expression, and prolonged calving intervals as well as reduced ovarian activity or true anestrus during heat stress (Singh *et al*., 2000). Furthermore, reproduction efficiency also was impaired by poor estrus detection and the variability of estrus duration (4-64 h) which led to the difficulty of predicting the exact or definite time of ovulation (Ohashi, 1994). It was evident that, only 34%- 49% of buffaloes exhibited estrus within the first 90 days post parturition, 31%-42% still anestrous for more than 150 days (El-Wishy, 2007). Moreover, anestrus due to ovarian inactivity is the cornerstone of buffalo's summer infertility, which limits the estrus behavior after parturition (Khan *et al*., 2012). Clinically, ovarian ultrasonography showed limited follicular growth, increased follicular atresia, complete absence of corpora lutea and pre-ovulatory follicles (Tanwar *et al*., 2003) attributed to malnutrition, suckling stress, low body score, and improper husbandry (Baruselli *et al*., 2001).

Principally, true anestrus is associated with low serum concentration of pituitary gonadotropins, and ovarian steroids (Terzano *et al*., 2012). Moreover, disappearance of LH surge (El-Wishy, 2007) is commonly observed at summer (De Rensis *et al*., 2007). Different attempts of treatment trials with variable results proposed to overcome postpartum true anestrus (Neglia *et al*., 2003; Azawi *et al*., 2012), which may be attributed to differences in synchronization protocols (Nayak *et al*., 2009; Naseer *et al*., 2012), ages and breeds variations (Derar *et al*., 2018), seasonal effects, and levels of nutrition (Nanda *et al*., 2003).

It is noteworthy that true anestrus buffaloes can be treated by vaginal progesterone-loaded devices (PRID) or ear implants of Norgestomet (Caesar *et al*., 2011; Naseer *et al*., 2012). Furthermore, Progesterone supplementations alone or combined with gonadotrophic drugs can restore follicular activities, ovulation induction with improved conception rates in the majority of buffaloes (Deka *et al*., 2010). Moreover, better performance is obtained by modified-synchronization progesterone programs, which improve the intra-follicular liquids, potentiate the pulsatory waves of LH, and its active receptors, in addition to the potent effect of E2 for perfect maturation of DF (de Carvalho *et al*., 2016). It was necessary to update the synchronization protocols by adding the intra-vaginal impregnated progesterone (PRID) in the Ovsynch protocol (Wiltbank *et al*., 2015) to achieve better reproductive outcomes of buffaloes. In the same way, Colazo *et al*. (2013) stated that Ovsynch protocol combined with PRID has positive effects on pregnancy rate, especially in anestrus cases with diminished serum progesterone at PGF2α administration. Moreover, the improved results of progesterone supplementations attributed to its negative feedback on the hypothalamus-pituitary-ovarian axis (Terzano *et al*., 2012). PRID removal exert dramatic decrease of blood progesterone concentration and enhances the high concentration of gonadotropin-releasing hormone (GnRH), FSH, LH, pulse frequencies, which sensibilize the hypothalamus to the action of Estrogens and restoring ovarian activity (Zerbe *et al*., 1999).

Unfortunately, Treatment of true anestrus buffaloes with the traditional methods didn't provide a radical solution to the problem, so there was a need to develop new methods to overcome the shortcomings of traditional methods. So, a promising alternative to overcome the disadvantages of GnRH analogues, such as low biological half-life, which evidently disappeared by modification toward the nano-drug delivery systems, which prolong the duration of efficacy of the biological compounds in circulation and at the cells of the target tissues with reduced therapeutic doses of GnRH analogues (Buserelin acetate) loaded on chitosan-tripolyphosphate (TPP) nanoparticles can enhance reproduction (Hashem and Gonzalez-Bulnes, 2020). Recently, the reproductive hormones can be loaded on naturally extracted Chitosan, which is a biodegradable, biocompatible compound, safe on living tissues, which can be used as a nanoparticle carrier with various routes of administration

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(Chen *et al*., 2013). In addition to, it possesses a positive surface charge, mucoadhesive properties to mucus membranes, penetrative enhancer, drug release to target organs, a continuous a source for drug release and delivery (Vårum *et al*., 1994).

In brief, loading different reproductive hormonal preparations to chitosan can increase the surge of GnRH, which improves the reproductive output without cytotoxic effects (Rather *et al*., 2013). According to the previously mentioned studies, this study was designed into two protocols: GnRH-PRID-synch protocol and GnRH-loaded chitosan-PRID-synch protocol in order to clarify the follicular dynamics, serum progesterone (P4), estradiol (E2) concentration, and conception rate in true anestrus buffaloes during the summer months.

Materials and methods

This study was designed (June to October) with an increased incidence of true anestrus buffalo cases. 50 multiparous lactating Egyptian buffalo cows (*Bubalus bubalis*), their average ages 5-8 years, and weighted 400- 450kg, average parity 3±0.2, and BCS 3-3.75 scale of Edmonson *et al*. (1989). Buffaloes are twice milked with an average daily milk production of 10.4±0.6 kg. These buffaloes have a history of normal parturition and uterine involution without signs of uterine pathology. Animals were reared in a confined stable with a half-covered shelter. The daily ration offered consisted of 40% concentrates, which contained 15 % crude protein, 0.5% Ca, 0.35% P, and 6.8 MJ/kg net energy level on a dry matter basis and 60% forages, including hay, straw, and darawa (whole green plant of corn), which was offered as green fodder, and water was offered ad lib.

Ethics approval

The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Diagnosis of true anestrus

True anestrus was diagnosed via two consecutive trans-rectal ultrasonography 10 days apart on day 85 and day 95 postpartum by SonoScape ultrasonography scanning (M12 Vet. Co. LTD, Shenzhen, China) with a linear-array transducer 5-7.5 MHz, which revealed the absence of the corpora lutea, and the dynamic follicular population (<10mm), with concurrent low serum progesterone concentration P4<1ng/ml (Navneet-Rohilla *et al*. 2005).

Experimental design

The second ultrasonographic scanning on day 95 postpartum (day zero of starting treatment), buffaloes divided to three groups based on absence of corpora lutea, active follicular growth (<10mm) on ovarian tissues and, low P4<1ng/ml. Groups were as follow:

GPP group (n=20, GnRH-PRID-synch protocol); On day 0, an elastomer coil (PRID) loaded with 1.55g of progesterone was inserted intravaginally for seven days (PRID, Ceva Santé Animale, Libourne, France), together with 2 ml Gonavet Veyx® 0.05 mg/ml Gonadorelin acetate intramuscular equivalent to 0.1 mg Gonadorelin as 6-D-Phe (Veyx-Pharma B.V. Forellenweg 16, NL-4941 SJ Raamsdonksveer, Netherlands). On day 7, PRID was removed and buffaloes received 2 ml Estrumate TM 250µg/ml/IM equivalent to 500 µg Cloprostenol (PGF2α, Vet Pharma Friesoythe GmbH, Germany). On day 9, buffaloes received 2 ml Gonavet Veyx® intramuscular, then buffaloes were artificially inseminated by fixed timed (FTAI) 16-20 hr later, as recommended by Barile *et al*. (2001) and Neglia *et al*. (2003)

GCP group (n=20, GnRH loaded on Chitosan-PRID-synch protocol); Gonadotropins were modified by loading GnRH on chitosan nanoparticles (GnRH-Ch-NPs) and combined with PRID as a source of exogenous progesterone for treatment of inactive ovaries and carried out in the same manner as the previous group, as follows: on day 0, buffaloes received only 1 ml of Gonavet Veyx® intramuscular equivalent to 0.05 mg/ml Gonadorelin modified and loaded on chitosan nanoparticles (GnRH-Ch-NPs) with intravaginal insertion of PRID loaded with 1.55g progesterone for seven days. On day 7, 2 ml Estrumate TM 250µg/ml intramuscular equivalent 500 mg Cloprostenol (PGF2α) and PRID were removed from the vagina. On day 9, buffaloes received the second dose of modified and loaded GnRH on chitosan nano-particles (GnRH-Ch-NPs) as 1 ml of Gonavet Veyx® intramuscular equivalent 0.05 mg /ml Gonadorelin, followed by FTAI at 16-20 hrs. later.

Control (n=10); Buffaloes received 10 ml intramuscular saline (NaCl 0.9%) on Days 0, 7, and 9, respectively. A fertile bull was introduced to detect the estrus in these buffaloes.

In general, buffaloes were inseminated on day 10 by FTAI with frozen-thawed semen of a known fertility buffalo bull from the International Center for Artificial Insemination, Al-Amriya, Alexandria, Egypt, frozen straw (1 ml) containing 20-30 X 106 sperm. Estrus signs and uterine tonicity felt as turgid, coiled, and edematous, as well as estrus mucus can be noticed, the cervix is relaxed, and ease of cervical passage during recto–vaginal insemination of buffaloes. Moreover, the size of the largest follicles was measured by ultrasonography at the same time of FTAI.

Hormonal assay

The serum concentrations of E2 and P4 were estimated using a specific ELISA Kit (Fine Test®, China) with 0.11 ng/ml and 8.2 ng/ml of lower concentration of P4 and E2, on arrangement. The intra-and inter-assay coefficients of variation were 9.3% and 9.9% for P4 and 9.3% and 8.2% for E2, respectively.also, the E2:P4 ratio was determined.

Preparation and properties of GnRH-loaded chitosan nanoparticles (GnRH-Ch-NPs)

According to the recommendations of Rather *et al*. (2013) and Hassanein *et al*. (2021), the ionic gelation method was used to fabricate chitosan nanoparticles (Ch-NPs) as nano-carrier for loading GnRH (GnRH-Ch-NPs) at faculty of agriculture, Alexandria University, Egypt. Meanwhile, the physicochemical properties of Ch-NPs, revealed the average particle size, and zeta potential were 62.13±0.17 nm, 0.347+28.0 mv, respectively and the similar criteria after loading GnRH–Ch-NPs (114.951.84 nm, 0.458+10.0 mv, respectively), with loading efficiency (LE%) 87.8% measured by zeta sizer model a NANO TRAC-WAVE II zeta sizer (MICROTRAC, USA) at scientific research and technological applications city, Alexandria, Egypt.

Ultrasonography and Reproductive Indices

Ultrasonography was used for diagnosis of ovarian inactivity on days 85 and 95 postpartum. Moreover, it was used for monitoring the follicular dynamics and the pre-ovulatory or the largest follicle at FTAI. Also, the occurrence of the ovulation on the 7th day after FTAI was detected through the disappearance of the largest follicles, in addition to the formation of the corpus luteum. The disappearance of the largest follicles and formation of CL on day 7 after FTAI was taken as a guide for occurrence of ovulation and to calculate the ovulation rate. Ovulation rate (%) was calculated.

Hormonal profile changes of P4 and E2 before and after application of protocols were assessed. Estrus phase clinical signs during rectal palpation (turgid, coiled, edematous, clear mucus, relaxed cervix, and ease of cervical passage) were taken as a criterion for response to treatment, and pregnancy rate after FTAI (%) was determined. Moreover, the pregnancy diagnosis by ultrasonography on day 35 post FTAI or introduction of a fertile bull in the control group.

Statistical analysis

All data were tested for normality by Shapiro-Wilk (W) test using the UNIVARIATE procedure (SAS, 2009) and the analysis revealed that all data was distributed normal (W > 0.90). Moreover, the data on follicular development, corpora lutea formation, and hormones concentrations were analyzed using the GLM procedure of SAS (2009) for testing the effect of treatment, time and their interaction. Besides, the significant differences between means were tested using Duncan multiple range test. Also, data on estrus induction, ovulation rate, and pregnancy rate were analyzed using a chi-square test at a confidence interval of 95%.

Results

It was clear that the highest progesterone concentration obtained on day 6. Also, a high concentration of P4 was observed in group GCP than GPP (Table1). Moreover, a sudden drop in P4 concentration on day 9 was prominent in GPP than GCP after PRID removal. Meanwhile, P4 concentration in control group didn't change over the days. The gradual increase of P4 concentration after PRID insertion appeared on day 3 but a higher concentration was noticed in GCP than GPP. It was cleared in GCP that P4 concentration was higher than GPP on day 3, and day 9, which may be attributed to the pattern of follicular dynamics, which may share in progesterone release, as well as the bioavailability of GnRH analogues loaded on chitosan nanoparticles. Furthermore, the higher concentration of estradiol was obtained on day 3, with higher values in GCP than in the other groups. Besides, the greatest concentration of estradiol was on days 9, and 12, with greater values in GCP, which may be attributed to enhanced or improved follicular growth.

Regarding Table 2, the ovarian follicular response improved with advancement of time. Also, the highest P2 and E2 were at day 7 and 9, respectively. Table 3 shows that GCP had the highest (P<0.05) CL size, followed by GPP, then control group. The same results were obtained for ovulation rate, which were 85.8% for GCP, 69.2% for GPP, and 10% for control group (P<0.05). Also, GCP group achieved the highest P4, and E2, concentrations, but the control group had the lowest. In addition, improved estrus induction and pregnancy rate in GCP group were noticed compared to other groups as recorded in Table 3.

Discussion

Inadequate follicular growth, absence of ovulatory follicles, and functional luteal structures represent the main features of buffaloes' true anestrus, which is principally predisposed by bad nutrition, heat stress,

Table 1. Averages of follicular size and serum P4 and E2 concentrations before and after starting treatments protocols.

Treatment protocol	[1]Day-10	[2]Day 0	Days of the experiment.			
			Day 3	Day 7	Day 9	Day 12
			Follicular Size mm			
GnRH-PRID-Synch	5.54±0.15^{Ad}	5.09±0.12^{Be}	5.89±0.15^{Bd}	7.03±0.11^{Bc}	8.41±0.21^{Bb}	12.46±0.14^{Ba}
GnRH-Ch-PRID-Synch	5.62±0.16^{Ade}	5.81±0.10^{Ad}	8.23±0.12^{Ae}	9.44±0.11^{Ac}	11.70±0.19^{Ab}	14.02±0.12^{Aa}
Control	5.41±0.20^{Ab}	5.23±0.24^{Ab}	5.76±0.15^{Ba}	5.29±0.16^{Cb}	5.54±0.18^{Cab}	5.57±0.20^{Cab}
Overall	5.54±0.095^{e}	5.40±0.090^{e}	6.79±0.185^{d}	7.64±0.237^{c}	9.15±0.352^{b}	11.70±0.457^{a}
			Progesterone ng/ml			
GnRH-PRID-Synch	0.55±0.03^{Ae}	0.68±0.03^{Ade}	2.61±0.12^{Ab}	8.19±0.20^{Ba}	1.70±0.11^{Bc}	1.16±0.07^{Ad}
GnRH-Ch-PRID-Synch	0.63±0.03^{Ad}	0.56±0.03^{Ad}	3.02±0.16^{Ab}	9.13±0.17^{Aa}	3.24±0.09^{Ab}	1.20±0.06^{Ac}
Control	0.55±0.03^{Ab}	0.59±0.06^{Ab}	0.55±0.05^{Bb}	0.57±0.05^{Cb}	0.56±0.06^{Cb}	1.57±0.91^{Aa}
Overall	0.58±0.019^{e}	0.61±0.020^{e}	2.36±0.154^{b}	7.04±0.477^{a}	2.08±0.158^{c}	1.26±0.180^{d}
			Estradiol.pg/ml.			
GnRH-PRID-Synch	1.85±0.08^{Ad}	1.56±0.08^{Ad}	5.56±0.14^{Bc}	10.27±0.73^{Bb}	12.72±0.94^{Ba}	10.29±0.65^{Bb}
GnRH-Ch-PRID-Synch	1.85±0.11^{Ae}	1.91±0.08^{Ae}	9.36±0.32^{Ad}	13.96±0.72^{Ac}	25.47±1.40^{Aa}	19.77±0.71^{Ab}
Control	1.65±0.11^{Ac}	1.67±0.14^{Ac}	6.06±0.18^{Bb}	7.65±0.87^{Cab}	8.77±1.50^{Ca}	6.93±0.65^{Cab}
Overall	1.81±0.058^{e}	1.72±0.057^{e}	7.18±0.293^{d}	11.22±0.559^{c}	17.03±1.239^{a}	13.41±0.860^{b}

$^{A-C}$ means with different letters in the same column under the same trait are differ (P<0.05). $^{a-c}$ means with different letters in the same row are differ (P<0.05).
PRID: progesterone releasing intra-vaginal device; Ch: chitosan nano particles; 1Day -10 Day 85 postpartum (at diagnosis day); 2Day 0 Day 95 postpartum (treatment starting day)

Table 2. Size of corpus luteum (CL), ovulation rate (%) and serum concentration of P4 and E2 on day 7 after insemination of different treatment protocols and control.

Item	Treatment protocols.		
	GnRH-PRID-Synch	GnRH-Ch-PRID-Synch	Control
CL size, mm	20.402±0.238^{b}	30.136±0.282^{a}	0.467±0.078^{c}
Ovulation rate, %	69.20%	85.80%	10%
P_4, ng. /ml.	2.480±0.246^{b}	2.964±0.274^{a}	0.731±0.155^{c}
E_2, pg./ml.	7.039±0.455^{b}	12.052±0.850^{a}	5.455±0.471^{c}

$^{a-c}$ Means with different letters in the same row are differ (P<0.05). CL = corpus luteum size.

Table 3. Estrus induction rate (%) and pregnancy rate (%) under treatment protocols and control.

Groups	Estrus induction rate % (frequency)			Total Estrus Induction rate %	Pregnancy rate %
	Day 1-6	Day 7-9	Day 10-12		
GnRH-PRID-Synch (n=20)	20-Jan	19-Feb	17-Jul	10/20 (50%)	8/20 (40%)
GnRH-Ch--PRID-Synch (n=20)	20-Feb	18-Jun	14-Sep	17/20 (85%)	14/20 (70%)
Control (n=10)	0/10	0/10	10-Jan	1/10 (10%)	0/10 (0%)

low BCS, and poor management (Sah and Nakao, 2010). In the present study, twice ultrasonography scanning of ovaries on days 85 and, 95 postpartum with ten days apart revealed that 29% of buffaloes without neither luteal structures, nor dominant follicular growth, were near to 31% reported by El-wishy (2007) and Azawi *et al.* (2012), 36.6% by Singh and Madan (1989), 41.2% by Roy *et al*. (1972). Moreover, 60% of summer true anestrus cases were reported as ovarian inactivity (Sah and Nakao, 2010). Also, 61.5% of true anestrus cases may extend 10-15 months, and 31.9% extend up to 16 months postpartum, attributed to hot weather (Sule *et al*., 2001). In the present study, true anestrus diagnosed by absence of prominent luteal structures and dominant follicular growth scanned by ultrasonography, associated with low serum progesterone and estrogen on day -10 (85) and day zero (95) respectively before beginning treatment, matched with Yotov *et al*. (2012) with an average basal progesterone level 0.5–1 ng/ml with an interval of 8–10 days later. Furthermore, several studies revealed that lower estradiol associated with inactive ovaries prevent LH surge secretion and estrus expression mainly due to in appropriate balance between estradiol and progesterone (Sarvaiya *et al*., 1993). In this study, low estradiol concentration before applying the treatment, then increased ($P<0.05$) on days 12 after treatment. These fundamental changes associated with fundamental increase ($P<0.05$) in serum concentration of progesterone on day 6 after PRID insertion with successful follicular growth to pre-ovulatory size. Numerous protocols like ovulation synchronization (Ovsynch) were used to manage true anestrus with limited improvement in reproductive efficiency up to 30% was achieved in buffaloes during the summer months (Azawi *et al*., 2012; Ghuman *et al*., 2014; Amin and Said, 2021). with low follicular growth during summer (Baruselli and Carvalho, 2005). This lowered efficiency of Ovsynch protocol because the GnRH depends on the follicular diameter and stage of development at administration (Rastegarnia *et al*. 2004), or due to the short biological half-life of GnRH (Amin and Said, 2021). Also, adequate follicular growth, increase atresia, and low sensitivity of luteal tissues to prostaglandin (Rastegarnia *et al*. 2004; De Rensis *et al*. 2005).Therefore, the disadvantages of Ovsynch disappeared when PRID included with GnRH and PGf2α (Zaabel *et al*., 2018) and better follicular turnover obtained after increased serum progesterone supplementation by PRID which sensitizes the hypothalamus-pituitary-gonadal axis once PRID removed (Baruselli *et al*., 2001), especially when combined with GnRH analogues and PGf2α (Peters, 2005) can effectively treat true anestrus of buffaloes. All studies stated that, intravaginal insertion of PRID for 7 days or more allows a dramatic increase in serum concentration of progesterone and exerts negative feedback on hypothalamus and anterior pituitary (Singh, 2003), promote GnRH, FSH and LH storage by adequate concentrations directly released once PRID is removed, with subsequent resumption of ovarian cyclicity (Zerbe *et al*., 1999). It is noteworthy that this mechanism was achieved in the present study, whereas the P4 concentration increased ($P<0.05$) in both protocols GPP and GCP than control on day 6 after insertion, but a higher level in GCP than GPP. However, dramatic drop in P4 concentration occurred on day 9 in both groups directly after PRID withdrawal. On the other hand, PRID included with Ovsynch protocols, can manage inactive ovaries of buffaloes during the low breeding season (Barile *et al*., 2001; Neglia *et al*., 2003), it has some shortcomings especially with the summer stress due to high temperature and humidity index. So, it was necessary to update and modify these programs to overcome low efficient GnRH analogs during hot conditions, which can be achieved by using a nano-drug delivery system (Archunan, 2020) which facilitates the delivery of drugs through circulation into the target sites such as loading GnRH analogs on chitosan tripolyphosphate nanoparticles (Rather *et al*., 2013; Hashem and Gonzalez-Bulnes, 2020; Amin and Said, 2021; Hassanein *et al*., 2021). Or by loading GnRH on chitosan dextran sulphate to different estrus synchronization protocols (Fernández-Serrano *et al*., 2017). Moreover, in the present study, the particle size of GnRH-loaded chitosan was reduced to 114.95 nm with loading efficiency 87.8%, and half the original dose of GnRH which is consistent with the results of Hassanein *et al*. (2021) and Gallab *et al*. (2022) who reported that loading GnRH on chitosan nanoparticles can reduce the original dose of GnRH to 50% without side effect on fertility performance of buffaloes during summer. Furthermore, loading GnRH to chitosan nanoparticles can extend GnRH biological half-life more than 5-6h with sustainable action, facilitate passage across tissue barriers, increase cellular uptake as well as bioavailability, and reduce GnRH dose to half of the conventional dose without antigenicity hazards. In the present study, the average follicular size before the beginning the treatment protocols agreed with the results of Amin and Said (2021) who noted that the follicular development doesn't exceed 5-6mm and the largest follicles do not exceed than 7mm before GnRH administration. Moreover, in the present study, a significant ($P<0.05$) follicular development toward dominance started once the progesterone effect was abolished on day7 until day12 in GPP, GCP protocols, and control, and these results coincide with Barile *et al*. (2001) and Neglia *et al*. (2003). It was evident that a higher P4 concentration in GCP than GPP buffaloes may be attributed to the sustainable action of GnRH analogs loaded on chitosan nanoparticles which promote follicular emergence and development of dominant follicular populations associated with a significant ($P<0.05$) estradiol concentration was increased in GCP followed by GPP compared to control buffaloes on day 9, which indicate the development of pre-ovulatory follicles, meanwhile, control group show a small size follicles on day12. It is noteworthy that, the prominent differences in GCP buffaloes were obtained by using half therapeutic dose as GnRH conjugated with chitosan nano-particles can provide sustained levels of FSH and LH which consequently promote adequate follicular growth and maturation (Rather *et al*.,2013) in addition to the high loading efficiency of chitosan biomolecules as delivery system (Bhattarai *et al*., 2006). Also, the enhanced results in GCP in terms of follicular sizes and associated steroid hormones were interpreted by Campanile *et al*. (2007) and Ramoun *et al*. (2012). Whereas, loading GnRH on chitosan nanoparticles can extend the biological half-life of GnRH and its efficacy, moreover, the perfect biocompatibility and biodegradability activities of loading GnRH on chitosan nanoparticles (Saranya *et al*., 2011). In the same regard, the summer ovarian inactivity can be treated when exogenous progesterone (PRID) is included with GnRH analogs like GnRH-PRID protocol (GPP) in the present study, which was reflected in the pattern of follicular turnover, development, hormonal and reproductive outcomes after PRID removal due to increased concentration of serum P4 in the blood (Rathour *et al*., 2005) were in agreement with the results of Barile *et al*. (2001) and Neglia *et al*. (2003). Furthermore, the modified GnRH-PRID-synch (GCP) in the current study was proposed as a new insight to collect the advantages of loading GnRH on chitosan nanoparticles beside the benefits of exogenous progesterone PRID and to overcome the unsatisfactory results of Ovsynch protocol (Neglia *et al*., 2003) for inactive buffaloes. it is evident that, the success of GCP and GPP programs relies mainly on the ability to induce a new follicular wave within a minimum temporal variation among animals, which was achieved by recent strategies such as loading GnRH analogs on chitosan nanoparticles to achieve enhanced induction of adequate and effective emergence of new follicular waves (Amin and Said, 2021). In the current study, the ovulation rate achieved (69.2%, 85.8% and 10%) was evident by disappearance of the pre-ovulatory follicles and formation of corpora lutea with different sizes (20.4mm, 30.1mm, and 0.467mm in GPP, GCP and control, respectively) on day 7 post FTAI. These results agreed with results of Murugavel *et al*. (2009); Oropeza *et al*. (2010) and Yotov *et al*. (2012). Regarding the conception rate in the present study, the obtained results of estrus induction (EIR) and conception rate (CR) were different to those obtained by Murugavel *et al*. (2009) obtained 47% EIR and 44.4% CR after application of PRID-synch protocols. Moreover, improved results of estrus induction rate and conception rate were recorded by Hiremath (2013). Also, a significant estrus induction rate of 90% (Oropeza *et al* 2010) and 62.5% (Warriach *et al*., 2008) were recorded when PRID was inserted with treatment protocols. This discrepancy may

be due to breed and nutrition factors (Barile *et al*., 1999). This response may be attributed to better circulating of E2 arising from pre-ovulatory follicles in GPP and GCP which agreed with (Herlihy *et al*. 2012). Furthermore, the present study achieved better E2/P4 proportion in GPP and GCP treated buffaloes with gradual increasing and concurrently with follicular development along days of implementation and consequently estrus induction was improved. The pregnancy rate in GPP of the present study were near to 44.5% reported by (Neglia *et al*., 2003) and 38.8% (Yotov *et al*., 2012) but lower than 65.2% (Mansour and Abdo, 1999) and 70.5 % (Presicce *et al*., 2005).

Overall, the superiority in the results of EIR and CR, follicular recruitment, and development of GCP in the present study may be attributed to improved loading efficiency of GnRH to 87.8%, delivering of GnRH loaded on chitosan nano-particles directed to ovaries (Fernández-Serrano *et al*., 2017, Hassanein *et al*., 2021), prolonged sustainability and bio-availability of GnRH release (Siahdasht, *et al*., 2020)

Conclusion

The conventional protocols manipulating the true anestrus are inefficient and do not provide a definitive solution to overcome summer reproductive insufficiency of buffaloes. Therefore, it was necessary to propose an innovative treatment protocols to overcome disadvantages of conventional treatment protocols, like loading GnRH on Chitosan nano-particles combined with PRID, which promote the follicular development toward the high potential fertilizing capacity, maturity and improve the reproductive performance parameters of buffaloes with ovarian inactivity when compared to GnRH-PRID-synch protocols, which may be attributed to the new acquired physicochemical properties of loading GnRH on chitosan nano-particles.

Conflict of interest

The authors have no conflict of interest to declare.

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