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Effect of breeding season and age on follicular dynamics and hemodynamics in embryo donor mares subjected to luteolysis after embryo flushing

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Abstract

Background: Mares are the only companion animals simulating women in the large diameter of their follicles. Horses start reproduction at the age of three years, and some of them live for >30 years, so aging influences their reproductive capacity. Mares are sensitive to summer heat stress as they can sweat like humans.

Aim: The current work aimed to study the effects of age (young versus senile), season (cold versus hot), and the hormonal treatments during embryo collection on the dominant and subordinate follicular dynamics and hemodynamics and circulating ovarian hormones in embryo donor mares ovulated twice spontaneously before inducing ovulation for flushing embryos.

Methods: Spontaneous oestrous cycles were studied for young mares (<10 years; $N = 6$) or senile (>20 years; $N = 5$) during months of the cold season (November to April) and hot season (May to August). In young embryo donor mares, oestrous cycles after inducing ovulation and luteolysis were studied using Doppler ultrasound. Estradiol (E2), progesterone (P4), nitric oxide (NO), total cholesterol, and lactate dehydrogenase (LDH) were measured in blood serum.

Results: A decrease in the dominant follicle antrum diameter ($p > 0.05$) and LDH ($p = 0.016$) was observed after inducing luteolysis in young embryo donor mares. Both human chorionic gonadotropin (hCG) and PGF2α treatments increased dominant follicle area ($p = 0.0001$), antrum area ($p = 0.001$), perimeter ($p = 0.001$), granulosa area ($p = 0.0001$), cholesterol ($p = 0.0001$), NO ($p = 0.0001$), and E2 ($p = 0.0001$). The dominant follicle area, antrum area, perimeter, color area, granulosa area, LDH, cholesterol, NO, and E2 increased ($p = 0.0001$) during the oestrous cycles of the hot season, but the circulatory % ($p = 0.0001$) declined. Senile mares had lower dominant follicle area ($p = 0.002$), antrum area ($p = 0.0001$), granulosa area ($p > 0.05$), LDH ($p = 0.001$), cholesterol ($p = 0.0001$), NO ($p = 0.0001$), and E2 ($p = 0.0001$) but higher circulatory % ($p = 0.0001$) and color area % ($p = 0.023$). The dominant follicle possesses the largest diameter, area, perimeter, granulosa area, and color area but the lowest circulatory % during spontaneous oestrous cycles, after inducing ovulation, or luteolysis with significant effects of the day of the spontaneous oestrous cycles on their dynamics and hemodynamics.

Conclusion: During hot months, mares treated with hCG ovulated 24 hours later and prostaglandin-induced luteolysis was followed by new ovulation five days later. Follicles ovulated during the hot months were larger than those ovulated during the cold months and both had nearly the same color area %. Senile mares ovulated follicles with a lower area and antrum area but a higher color area %, so senile mares can be used as embryo or oocyte donors during the hot season.

Keywords: Embryo transfer donors, Follicular dynamics, Induction of ovulation, Mares, Season.

Introduction

The oestrous cycle of Arabian mares determined by the return to mounting and the observation of oestrous behavioral signs of brood mares before the use of ultrasound reported that the regularity of oestrous all year round had different lengths with little or no significant seasonal variations in Egypt (El-Ghannam and El-Sawaf, 1967). In mares, the change in follicular blood vascularization using either color or power Doppler assessment depended on the day before the selection to the deviation of the dominant and the subordinate follicles (Abdelnaby and Abo El-Maaty, 2017a,b). During the spontaneously ovulating oestrous cycle, the follicular dynamics and vascularization

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vary between the dominant or subordinate follicles until ovulation (Aboelmaaty and Abdelnaby, 2017). Changes in the ovarian vascular network during estrus allow the delivery of essential components for follicular maturation and steroidogenesis (Watson and Al-Ziábi, 2002).

Several hormones have been tried to synchronize estrus and ovulation in mares. Bollwein *et al*. (2004) administered mares' exogenous progesterone for 14 days after ovulation and estradiol benzoate three times at five-day intervals from the day of ovulation. They noticed an improvement in the blood flow of uterine arteries and to the dominant ovarian. Regardless of the ovsynth protocol commonly used to synchronize estrus in cattle, the Ovsynth protocol was not recommended to synchronize estrus in mares (Glazar *et al*., 2004). GnRH was used to hasten ovulation in mares with an oestrous follicle >30 mm in diameter (Stich *et al*., 2004). Human chorionic gonadotropin (hCG) was used at the end of most synchronization protocols to synchronize ovulation in mares (Bristol, 1993) by increasing follicular vascularization and left uterine horn (LH) concentrations for 24 hours after treatment (Boakari *et al*., 2017). During either spontaneous or induced ovulations using hCG, the follicle vascularity did not increase during the first 30 hours after treatment, and the follicle vascularity remained constant and low during the last six hours before ovulation (Boakari *et al*., 2017). After hCG treatment, the thickness and echogenicity of the granulosa and the color-Doppler end-points increased. Still, the prominence of an anechoic band, color-Doppler signals, and estradiol decreased in treated and non-treated mares by approaching ovulation a few hours before ovulation (Gastal *et al*., 2006).

Nitric oxide (NO) has the advantage of being a physiological component in regulating blood flow in the genital tract (Musicki *et al*., 2009). The pulsatility (PI) index of both uterine arteries and the dominant ovarian artery declined in mares who received NO donner (30 or 60 mg of isosorbide dinitrate) compared with placebo-treated mares, indicating increased blood flow in the dominant ovarian artery in treated pregnant mares than in placebo-treated pregnant and cycling mares (Zoller *et al*., 2016). Isosorbide dinitrate increased uterine and ovarian perfusion in cycling mares and ovarian perfusion in early pregnant mares (Zoller *et al*., 2016).

The age of the mares does not affect systemic estradiol concentrations, the diameter of the preovulatory follicle, and the color-Doppler signals of blood flow in the follicle wall before ovulation (Ginther *et al*., 2009). Egypt was declared one of the most vulnerable countries to climate change's potential impacts and risks, as stated in the Second National Communication to the United Nations Framework Convention on Climate Change (EEAA, 2010). In mares, the effect of summer heat stress on ovarian hormones and follicular hemodynamics was not studied. However, the effect of increased body temperature as a result of 30 minutes of exercise for six days per week resulted in lower recovered embryo quality (Mortensen *et al*., 2009), increased cortisol, lowered LH, delayed deviation, and elongated inter-ovulatory intervals (Kelley *et al*., 2011). The temperature-humidity index (THI) was used to indicate heat stress was associated with an increased percentage of early embryonic loss after the transfer of embryos in recipient mares "(Vasquez *et al*., 2010; Yu *et al*., 2022). Still, the decline in the pregnancy rate in commercial embryo recipient mares was referred to as seasonal effects, not high THI (Fanelli *et al*., 2022). All body cells produce lactate dehydrogenase (LDH) enzymes and their activity increases during damage and death of the cells. Its activity varies in the ovarian follicles according to the atresia classification day of the oestrous cycle. It was reported to increase the follicular fluid of the dominant buffalo ovarian follicles compared to small-sized ones (Abd Ellah *et al*., 2010). In equine, LDH activity declined in serum and the follicular fluid of the large ovarian follicles >30 mm compared to the small ones (Satué *et al*., 2019). Cholesterol is the precursor of steroid hormones and is one of the main components of follicular fluid and blood serum for the steroidogenesis of sex hormones. In cyclic mares, cholesterol concentrations increased in the follicular fluid compared to blood serum, and with the increase of the diameter of the ovarian follicles from 20.0 to >40 mm (Satué *et al*., 2019). However, cholesterol concentrations were nearly similar in large and medium size follicles of buffaloes (Abd Allah *et al*., 2010). This study explored the effects of summer heat stress, age, and hormonal protocols used during embryo recovery programs on dominant and subordinate follicle dynamics and hemodynamics, ovarian hormones, NO, and LDH in Egyptian mares.

Materials and Methods

Animals, housing, and feeding

Clinically healthy, non-lactating, non-pregnant, and normally cycling mares were used in the current work. They were divided into two groups. Old mares (N = 5; of 20–26 years old) were granted from the Police Horse department for research. Young mares (N = 6; 6–10 years old) were purchased from private horse studs. Mares were kept on the research farm of the Theriogenology Department, Faculty of Veterinary Medicine, Cairo University. Animals were kept in an open yard during the day and indoors at night with artificial light. All animals were dewormed regularly and received medications for blood parasites. The study was conducted from November 2022 to August 2023.

Ultrasonographic (US) and Doppler examinations

The ultrasound-Doppler scanner equipped with a 12 MHz transrectal linear-array trans-rectal transducer (Sonovet R3, Madison, Samsung, South Korea) was used to examine ovarian and uterine hemodynamics.

The numbers and diameters of ovarian follicles were determined throughout the study period. Daily examinations were done once the dominant follicle reached 30 mm in diameter, combined with uterine oedema and oestrous behavior. The follicular hemodynamics were determined from November to April (cold season) and May to August (hot season) using the THI = 0.8 × Temp. °C+ Relative humidity % × (Temp. °C −14.4) + 46.4 (Diaz *et al*., 2023). Table 1 presents the monthly changes in Cairo's maximum environmental, relative humidity percentage, THI, and risk evaluation. A THI of ≤74 or less is considered normal, 75 to ≤79 is alert status, 80 to ≤84 is danger status, and the THI equal to or above 84 is an emergency (Fanelli *et al*., 2022).

The day of ovulation (Day 0) was designed as the day when the largest and dominant follicle achieved the maximum diameter and then disappeared or sharply decreased in diameter on the following day (Abo El-Maaty and Abdelnaby, 2017). The *corpus luteum* diameter was determined from its first appearance till the approach of the next ovulation (Abdelnaby and Abo El-Maaty, 2017b). In addition, the *corpus luteum* hemodynamics was recorded on each examination after ovulation using the color Doppler mode.

The ovarian arteries' diameters, blood flow velocities, and blood flow indices were determined using the pulsed-wave Spectral Doppler mode (Bollwein *et al*.2004). Moreover, the diameters and hemodynamics of the uterine body and horns were determined (Abdelnaby *et al*., 2016).

Ovarian and uterine hemodynamics

The ovarian follicular dynamics and hemodynamics were determined for all animals from day 6 before ovulation till day 14 (spontaneous ovulation) throughout the oestrous cycles from November to June. All mares were subjected to induced ovulation using hCG for embryo recovery (Abdelnaby and Abo El-Maaty, 2017a, b). The day of ovulation (Day 0) and the *corpus luteum* formation were evaluated till the day of embryo flushing (Day 7 and Day 8). Induced luteolysis started the next day of the embryo flushing upon using a luteolytic drug. The diameter of the dominant and subordinate follicles and their antral cavities were recorded (Abdelnaby and Abo El-Maaty, 2017a). The vascularization area of the dominant and subordinate (Fig. 1A, B, F) ovarian follicles also was recorded (Abo El-Maaty and Abdelnaby, 2017). The granulosa area was estimated by subtracting the antrum area from the follicle area. The estimation of follicular vascularization percentage was calculated by dividing the color area/pixel on the follicle area/pixel. The peak systolic velocity, the end-diastolic velocity (END), and the time average mean velocity were determined (Abdelnaby *et al*., 2018). In addition, the PI and resistance (RI) indices and the blood flow volumes were recorded for every ovarian and uterine artery (Bollwein *et al*., 2004).

The day of ovulation was confirmed when the most enormous and prevailing follicle achieved a diameter ≥35 mm in diameter before disappearing on the following Day (Abo El-Maaty and Abdelnaby, 2017). The luteal (Fig. 1C and D; Abdelnaby and Abo El-Maaty, 2017b), uterine body, and uterine horns diameters and hemodynamics (Fig. 1E), in addition to the uterine arteries blood flow indices were determined (Abdelnaby *et al*., 2016). The uterine assessment before embryo flushing was performed to confirm the absence of subclinical or clinical endometritis (Abdelnaby *et al*., 2016**;** Bollwein *et al*., 2004). The images and video clips were recorded on the ultrasound and then were exported using a portable removable disk for later analysis using image analysis software.

Embryo recovery

For recovering embryos, mares were inseminated with fresh semen from Arabian stallions of proven fertility

Table 1. Average environmental temperature, humidity, and photoperiod during the study period in Cairo.

Month	Max. temp. °C	Min. Temp °C	Photoperiod/h	Humidity %	THI	Risks
November	26	13	10.7	56	73.70	Normal
December	21	9	10.3	58	67.03	Normal
January	19	7	10.4	55	64.13	Normal
February	22	8	11.1	49	67.72	Normal
March	26	10	12	45	72.42	Normal
April	30	13	12.9	39	76.48	Alert
May	34	17	13.6	78	88.89	Emergency
June	37	20	14	83	94.76	Emergency
July	39	21	13.9	42	87.93	Emergency
August	39	22	13.2	44	88.42	Emergency
September	36	20	12.4	48	85.57	Emergency
October	32	17	11.5	53	81.33	Danger

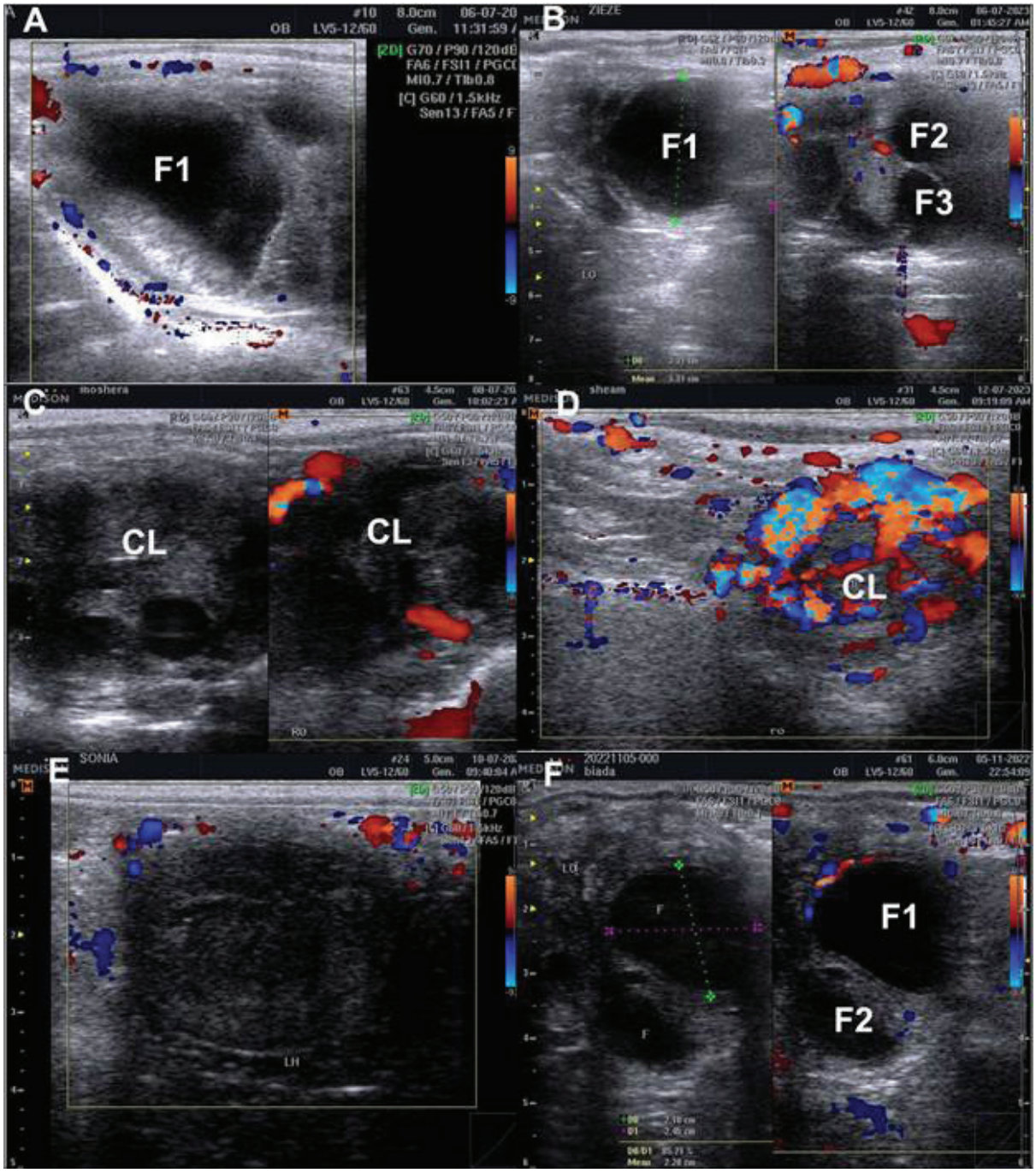

Fig. 1. Ultrasonograms show the dominant follicle (F1) at hCG administration in young donor mare (A). F1 at hCG administration in senile donor mare (B). Developed corpus luteum (CL) two days after hCG (C). Mature CL six days after hCG before embryo flushing (D). LH color blood vascularization (E). dominant follicle during cold season.

(Sieme *et al*., 2001). Mares were inseminated 24 and 48 hours after administering 2,500 iu hCG (Epifasy, EIPICO, Egypt). Uterine flushing of all mares was performed 7 and 8 days after insemination, with day 0 being designated the day of ovulation (Squires *et al*., 2003).

After embryo flushing, each mare was injected with 10 mg Dinoprost tromethamine (Lutalyse, Zoetis, Belgium SA) to discontinue possible pregnancies resulting from unrecovered embryos (Pessoa *et al*., 2011; Campbell, 2014). Mares were examined before and after embryo flush and from the following day

till the next dominant follicle reached >30 mm in diameter, then prepared for another round of embryo recovery.

Blood sampling

Immediately before each US examination, a blood sample was collected from the jugular vein of each mare and placed into two tubes: a tube containing EDTA as an anticoagulant for assessment of hormonal profiles (Progesterone, Estradiol) in plasma and another serum tube for NO, biochemical oxidants, and antioxidants analysis. All samples were immediately placed on ice and centrifuged (10 minutes at 3,000 r.p.m) within 2 hours after collection. Plasma and sera were stored at −20°C until they were assayed.

Hormone assaying

Progesterone and estradiol hormones were assayed using a commercial ELISA kit (DRG Instruments GmbH, Germany). For progesterone, the range of the assay was between 0.0 and 40 ng/ml, the sensitivity was 0.045 ng/ml, and intra- and inter-assay variability was 6.81% and 7.25%, respectively. For estradiol, the range of the assay was between 9.7 and 200 pg/ml, the sensitivity was 9.714 pg/ml, and intra- and inter-assay variability were 6.86% and 5.59%, respectively. LDH (NS 283001) was assayed using a colorimetric kit (Salucea, Dutch technology in life science, Haansberg 19, 4874 NJ Etten Leur, The Netherlands). Total cholesterol was assayed using a colorimetric kit (MG, Science and Technology Center "STC," Egypt). NO was assayed using a colorimetric kit (Biodiagnostics, Egypt).

Statistical analysis

Data were expressed as Mean ± SEM. Analysis of variance (ANOVA) was used for the sequential data using software (SPSS, 2016). Duncan's Multiple Range test was used to differentiate between significant means at $p < 0.05$. The effect of age (young versus senile), season (cold versus hot), and days during spontaneous ovulation (Days 6 to 14), induced ovulation (Days 1 to 8), and induced luteolysis (Days 0 to 24) on dominant and subordinate follicles diameter, antrum diameter, area, antrum area, color area, granulosa area, color area %, and granulosa area % were studied using independent sample *t*-test for determining age and season effects and simple One-Way ANOVA to study the impact of days within spontaneous and induced ovulations and after inducing luteolysis. Duncans' Multiple Range test was used to differentiate between significant means at $p < 0.05$.

Ethical approval

The study protocol was approved by the Animal Care and Ethical Use Committee of the Faculty of Veterinary Medicine at Cairo University (Approval ID: Vet-CU-03162023708).

Results

The diameter of the dominant follicle insignificantly increased shortly after inducing ovulation (Table 2). Dominant follicles developed after inducing luteolysis tended ($p > 0.05$) to have smaller antrum diameters. The dominant follicle area, antrum area, perimeter, and

Table 2. Mean ± SEM of dominant follicle dynamics and hemodynamics and the circulating E2, P4, total cholesterol, NO, and LDH enzyme (U/L) in mares spontaneously ovulated, mares induced to ovulate for embryo flushing, and mares subjected to induced luteolysis after embryo flushing.

Dominant follicle	Spontaneous	Induced ovulation	Induced lutealysis	*p* value
Diameter/cm	3.28 ± 0.08	3.57 ± 0.15	3.21 ± 0.29	0.207
Antum diameter/cm	2.32 ± 0.07^{b}	2.59 ± 0.14^{b}	2.05 ± 0.26^{a}	0.086
Area/pixel	45770 ± 2611^{a}	70288 ± 5163^{b}	68282 ± 9269^{b}	0.0001
Antrum area/pixel	24970 ± 1613^{a}	39427 ± 3980^{b}	39820 ± 6667^{b}	0.001
Circulatory %	73.00 ± 0.60	71.54 ± 1.33	73.36 ± 1.91	0.537
Perimeter/pixel	872 ± 32.83^{a}	1094 ± 42.91^{b}	1050 ± 87.46^{b}	0.001
Color area/pixel	3709 ± 463	4652 ± 1131	4672 ± 1366	0.608
Granulosa area/pixel	20389 ± 1241^{a}	32355 ± 2696^{b}	28460 ± 3127^{b}	0.0001
Color area%	8.07 ± 0.69	6.76 ± 1.26	7.13 ± 2.05	0.659
Granulosa color area%	16.32 ± 2.47	14.94 ± 3.34	14.79 ± 3.60	0.951
LDH U/L	34.17 ± 3.48^{ab}	56.39 ± 10.30^{b}	23.13 ± 11.88^{a}	0.016
Cholesterol mg/dl	142.04 ± 3.78^{a}	181.46 ± 2.93^{b}	187.95 ± 4.98^{b}	0.0001
NO µmol/L	32.23 ± 1.39^{a}	53.47 ± 2.53^{b}	48.99 ± 1.18^{b}	0.0001
E2 pg/ml	59.99 ± 3.63^{a}	82.63 ± 3.91^{b}	48.99 ± 1.18^{a}	0.0001
P4 ng/ml	7.78 ± 0.42	6.51 ± 0.71	6.24 ± 1.30	0.208

Means with different superscripts (a,b) between columns are significantly different at $p < 0.05$.

granulosa color area increased ($p < 0.001$) after inducing ovulation using hCG and inducing luteolysis using prostaglandin. Compared to the induced ovulation, the induction of luteolysis declined ($p < 0.05$) LDH (Table 2). Both induction of ovulation and induction of luteolysis increased ($p < 0.0001$) total cholesterol, estradiol (E2), and NO.

Compared to the cold season, a non-significant increase was recorded in the follicle diameter, antrum diameter, color area %, and granulosa color area % during the hot season (Table 3). The dominant follicle area, antrum area, perimeter, color area, and granulosa area increased ($p < 0.001$), but the circulatory % decreased ($p < 0.0001$) during the hot season (Table 3). Moreover, LDH, Total cholesterol, NO, and E2 increased ($p < 0.0001$) during the hot season (Table 3). Dominant follicles of senile mares (Table 3) had a lower area ($p < 0.01$), antrum area ($p < 0.0001$), and tended to have lower ($p > 0.05$) granulosa area but possessed higher circulatory % ($p < 0.0001$) and color area % ($P < 0.05$). Senile mares have lower LDH ($P < 0.001$), total cholesterol, NO, and E2 ($p < 0.0001$).

The days during the spontaneous ovulations influenced the follicle diameter ($p < 0.0001$; Fig. 2A), antrum diameter ($p < 0.0001$; Fig. 2B), follicle area ($p = 0.035$; Fig. 2C), antrum area ($p = 0.016$; Fig. 2D) circulatory ($p < 0.013$; Fig. 2F), color area % ($p = 0.027$; Fig. 3C); and tended to influence the granulosa area ($p > 0.05$; Fig. 3B). A significant increase in the dominant follicle diameter ($p < 0.0001$; Fig. 2A), antrum diameter ($p < 0.0001$; Fig. 2B), area ($p = 0.035$; Fig. 2C), antrum area ($p = 0.016$; Fig. 2D), insignificantly high perimeter (Fig. 2E), and significantly low circularity % ($p < 0.013$; Fig. 2F) compared to the subordinates throughout the spontaneous ovulation oestrous cycle.

During the spontaneous ovulations, the mean follicle dominant and subordinates' diameters (Fig. 2A) were the lowest on day 8 (1.46 ± 0.13) and the highest on days 1 (3.39 ± 0.27) and day 0 (Day of ovulation; 3.37 ± 0.21) that sharply declined on day 1 to reach (1.79 ± 0.52). Meanwhile, the dominant follicle's diameter (Fig. 2A) and the antrum diameter (Fig. 2B) keep increasing from Day 6 to reach the highest values on the day before ovulation. Those subordinate follicles start decreasing from day 5 to get the lowest value on day 3, then increase for two days with a lower growth rate until they reach the day of ovulation (Day 0). The dominant follicle area (Fig. 2C), antrum area (Fig. 2D), and perimeter (Fig. 2E) increased from day 3 to reach the maximum values on the day before ovulation. The circulatory % (Fig. 2F) of the dominant follicles decreased as approached ovulation except for the day before ovulation; the largest subordinate achieved the highest circulatory % on the day of ovulation. The color area of the dominant follicle increased significantly on day 4 and day 3, but that of the largest subordinate increased sharply on day 5 (Fig. 3A). The granulosa area of the dominant follicle became maximum on day 1 and day 4. Still, those of the largest subordinate peaked on day 2 and day 5 (Fig. 3B). The color area % (Fig. 3C). and the granulosa color area % (Fig. 3D) of the dominant and largest subordinate increased sharply on day 5 and declined to the lowest value on day 0 (Fig. 3C).

Table 3. Mean ± SEM of dominant follicle dynamics and hemodynamics and the circulating E2 (pg/ml), P4 (ng/ml), total cholesterol (mg/dl), NO (µmol/L), and LDH enzyme (LDH U/L) in young and senile mares and those spontaneously ovulated during hot (non-breeding) and cold (breeding) seasons.

Dominant follicle	Cold season	Hot season	*p*-value	Senile	Young	*p*-value
Diameter/cm	3.31 ± 0.11	3.46 ± 0.09	0.325	3.38 ± 0.09	3.42 ± 0.12	0.810
Antum diameter/cm	2.43 ± 0.10	2.37 ± 0.08	0.628	2.40 ± 0.08	2.38 ± 0.11	0.864
Area/pixel	33663 ± 2143	65405 ± 3657	0.0001	46321 ± 3121	62815 ± 4454	0.002
Antrum area/pixel	18182 ± 1467	35584 ± 2485	0.0001	23320 ± 1768	36184 ± 3121	0.0001
Circulatory %	75.56 ± 0.74	71.18 ± 0.82	0.0001	75.18 ± 0.61	69.88 ± 1.03	0.0001
Perimeter/pixel	595 ± 34.63	1016 ± 32.71	0.0001	934 ± 50.23	991 ± 40.80	0.390
Color area/pixel	2546 ± 221	5262 ± 699	0.0004	4809 ± 739	3539 ± 452	0.168
Granulosa area/pixel	14420 ± 1134	30295 ± 1705	0.0004	22420 ± 1834	26982 ± 1780	0.08
Color area%	8.06 ± 0.59	8.25 ± 0.94	0.889	9.50 ± 0.97	6.64 ± 0.72	0.023
Granulosa color area%	15.18 ± 4.85	17.73 ± 1.99	0.575	18.42 ± 3.79	14.86 ± 1.61	0.417
LDH U/L	22.72 ± 1.56	50.31 ± 4.17	0.0001	27.00 ± 1.95	50.67 ± 4.83	0.001
Cholesterol mg/dl	107.99 ± 1.11	182.41 ± 1.37	0.0001	119.57 ± 2.28	182.13 ± 1.54	0.0001
NO µmol/l	20.17 ± 0.38	50.49 ± 0.88	0.0001	24.59 ± 0.85	51.12 ± 1.04	0.0001
E2 pg/ml	53.92 ± 3.23	75.43 ± 1.91	0.0001	54.46 ± 2.81	79.78 ± 1.97	0.0001
P4 ng/ml	7.26 ± 0.34	7.20 ± 0.34	0.507	7.40 ± 0.30	6.92 ± 0.39	0.224

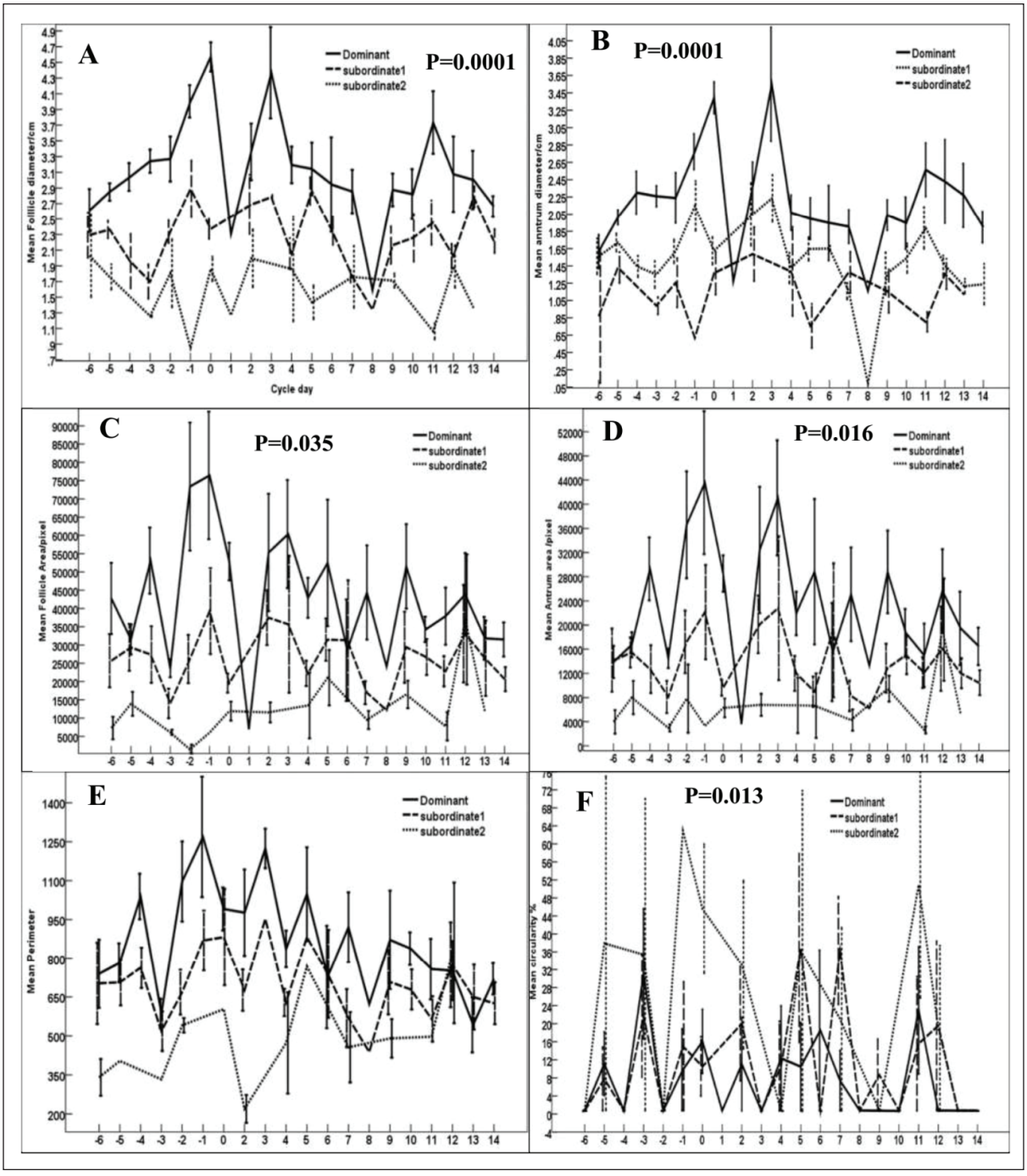

Fig. 2. Dominant and subordinate follicles values evaluated during cycles where ovulations were spontaneous. The diameter/cm (A) and (B) the antrum diameter/cm, area/pixel (C), antrum area/pixel (D), perimeter/pixel (E), and circulatory % (F) with SEM bars. Ovulation is Day 0.

Days after hCG did not influence the dominant follicle diameter and area, the antrum diameter and area, the perimeter, and the circulatory, The follicle area and follicle color area %, and the granulosa area and the granulosa color area %. Though hCG increased the dominant follicle diameter (Fig. 4A), antrum diameter (Fig. 4B) for two days, and the circulatory % (Fig. 4F) for one day after administration, the dominant follicle area (Fig. 4C), antrum area (Fig. 4D), the perimeter (Fig. 4D), and the granulosa color area (Fig. 5B)

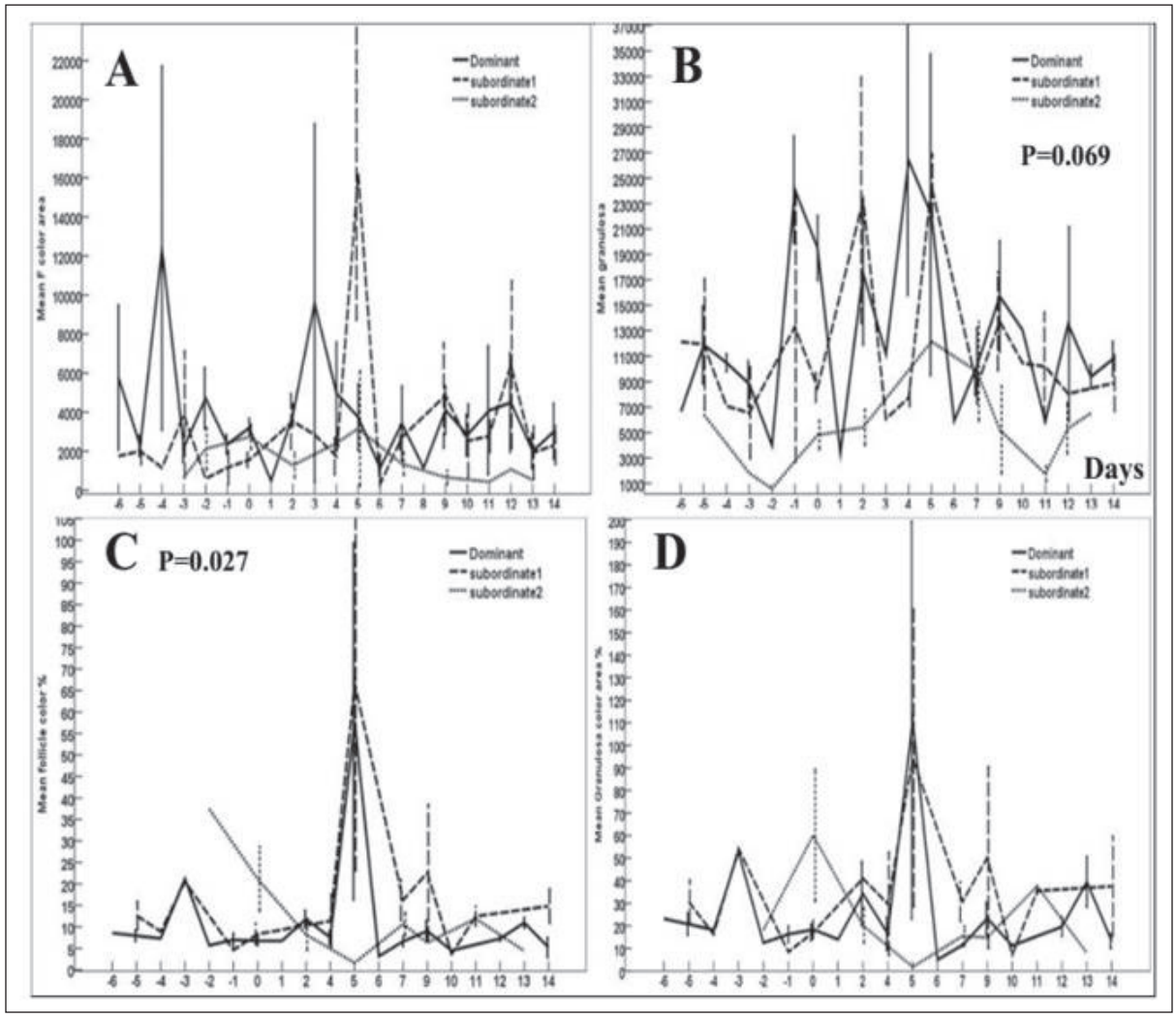

Fig. 3. Dominant and subordinate follicles (first and second largest) values evaluated during cycles where ovulations were spontaneous. Day 0: day of ovulation. (A) Follicle color area/pixel, (B) follicle granulosa area/pixel, (C) follicle color area %, and (D) granulosa color area % with SEM bars. Ultrasonogram showing the dominant follicle at (E) hCG administration and (F) ovulation confirmed two days later.

declined linearly along 48 hours. The dominant follicle color area (Fig. 5A), color % (Fig. 5C), and granulosa color area % (Fig. 5D) declined sharply 24 hours after hCG.

The administration of PGF2α after embryo flushing showed an increase in the dominant follicle diameter (Fig. 6A), antrum diameter (Fig. 6B; $p < 0.05$), area (Fig. 6C), antrum area (Fig. 6D), perimeter (Fig. 6E), granulosa area (Fig. 7B), and an associated decrease in the circulatory % (Fig. 6F), follicle color area % (Fig. 7C; $p = 0.035$), and granulosa color area % (Fig. 7D) on Day 5 and day 24 after administration.

Discussion

The induction of ovulation in donner mares subjected to embryo flushing and the induction of luteolysis on day 8 and day 9 (Days of embryo flushing in this study had no influence on follicle diameter, but both treatments significantly increased follicle area and antrum area). In contrast, the administration of GnRH on day 8 or day 12 after ovulation and prostaglandin on days 15, 6, 15, 10, and 19, though all mars received altrenogest for ten days starting from day 5 after ovulation, did not induce luteolysis or ovulation of diestrus follicles after GnRH administration (Glazar *et al*., 2004). Similar to hCG, deslorelin-induced ovulation within two days in mares having follicles >3.4 cm in diameter with no difference in the number of large follicles 14–15 days after ovulation compared to control mares (Stich *et al*., 2004).

Contrary to the induced ovulation five days after embryo flushing performed eight and nine days

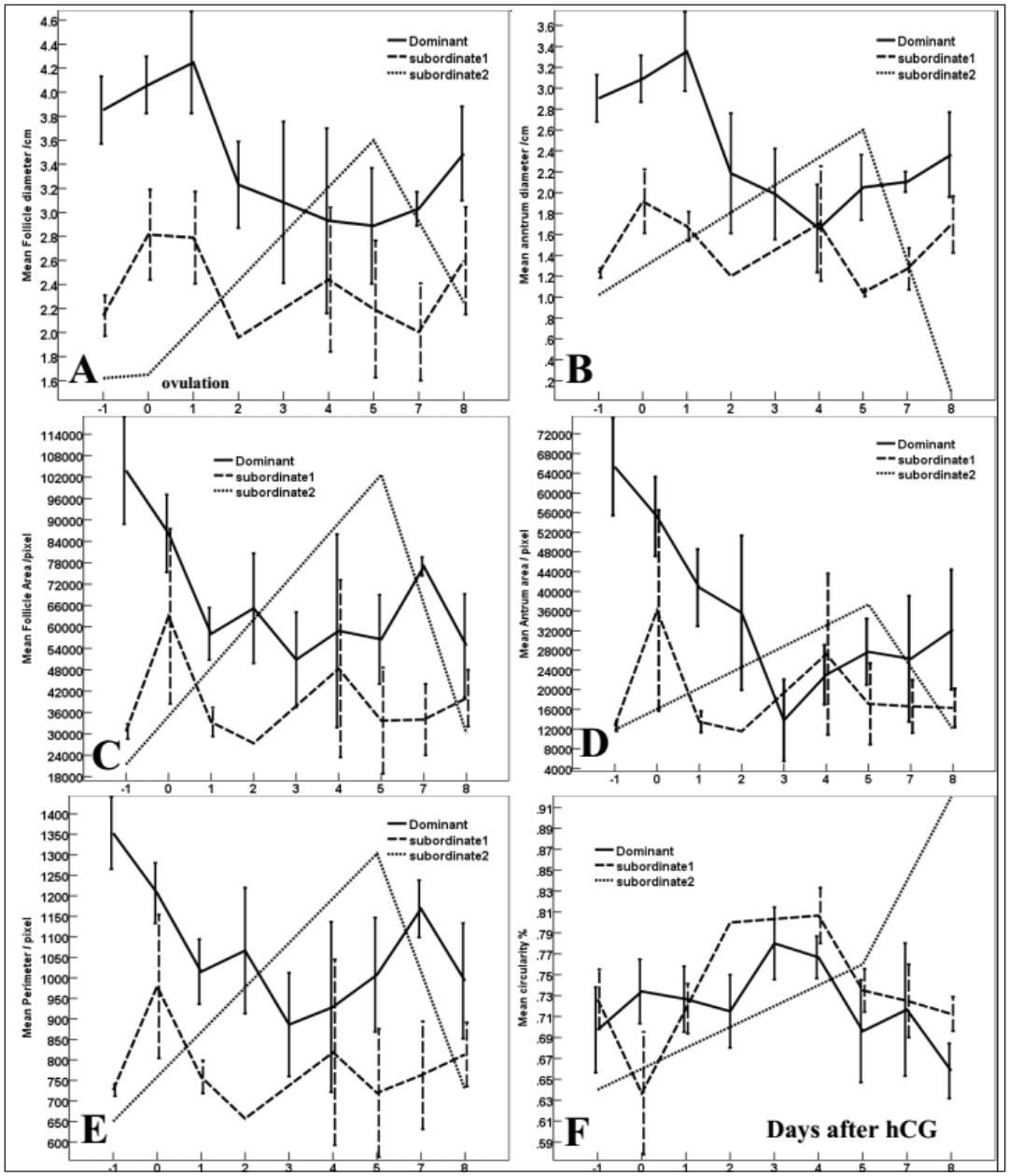

Fig. 4. Dominant and subordinate follicles (first and second largest) values evaluated during cycles where ovulations were induced with hCG. (A) Follicle diameter in cm, (B) follicle antrum diameter in cm, (C) follicle area in pixels, (D) follicle antrum area in pixels, (E) follicle perimeter in pixels, and (F) circulatory % with SEM bars.

after hCG treatment followed by PGF2α treatment on the day of embryo flushing in our study, cycling mares ovulated 7.8 ± 2.5 days after PGF2α treatment (Zoller *et al*., 2016). The increase in the color area indicated decreased blood flow RI; the reverse is true. In agreement with the increased blood flow RI on day 1 and from day 6 to days 10 to 11, followed by a decrease from day 2 to days 4 to 5 that increased on day 9 in the dominant ovarian and uterine arteries, mares spontaneously ovulated in our study showed an increase in the dominant and subordinate follicular blood flow color area from days 2 to 5 during their selection for the next cycle ovulation and from day 5 to day 8 after hCG treatment and day 14 after PGF2α treatment (Zoller *et al*., 2016).

In mares, circulating estradiol concentrations reach a maximum of about two days before ovulation (Ginther *et al*., 2008). The preovulatory estradiol surge begins to decrease three days before the peak of a prolonged LH surge (about seven days) in mares (Ginther *et al*.

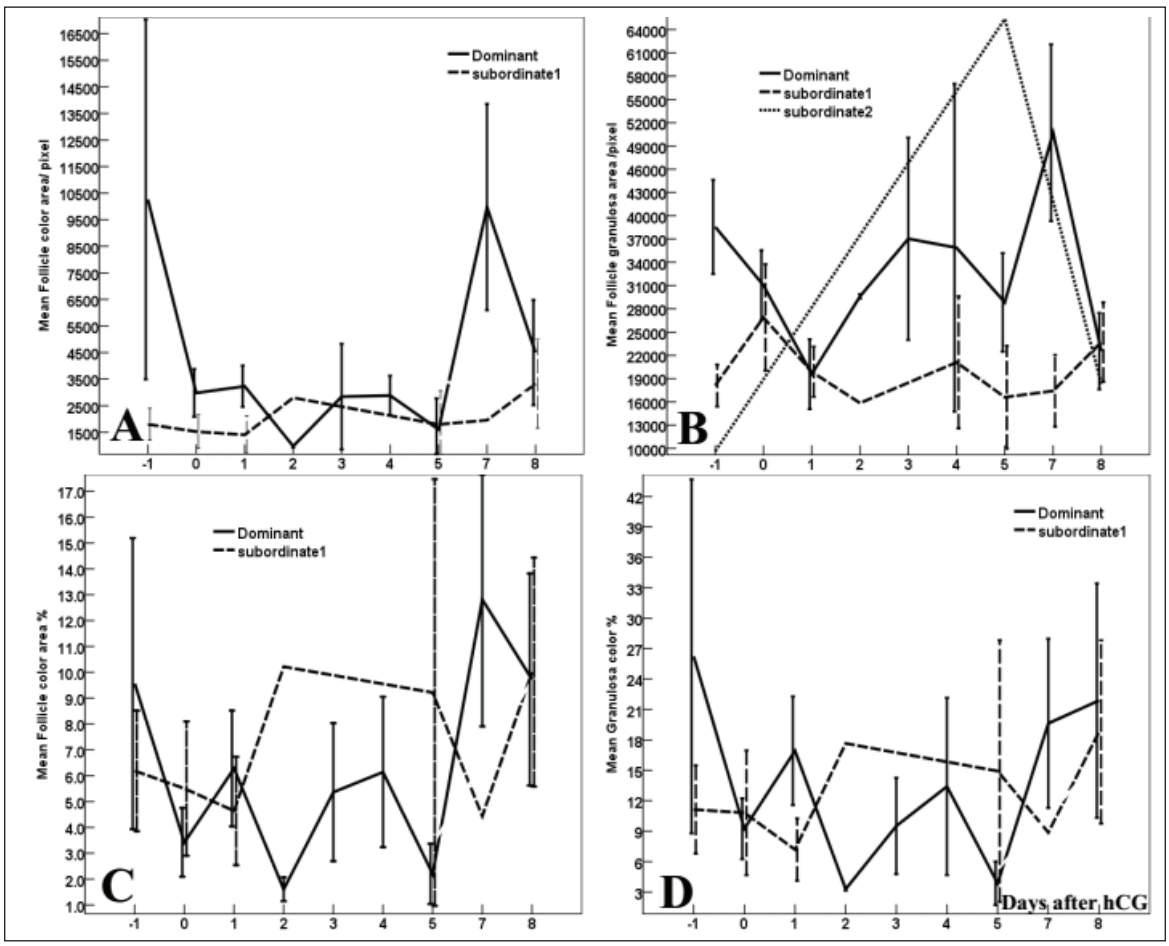

Fig. 5. Dominant and subordinate follicles (first largest) values evaluated during cycles where ovulations were induced with hCG. (A) Follicle color area in pixels, (B) Follicle granulosa area in pixels, (C) Follicle color area %, and (D) Follicle granulosa color area % with SEM bars.

2008). Concentrations in the follicular fluid have been reported to decrease (Gérard *et al*. 1999) and not decrease (Watson and Sertich 1991) before ovulation.

Days 15–17 (Day 0 = ovulation) was defined as the luteolytic period. The mean percentage of corpus luteum (CL) with color-Doppler signals for blood flow was maximum on day 10 (77.3%), and days 10–14 (49.8%) were defined as the pre-luteolytic period. The cross-sectional area of the CL decreased progressively from day 4 (9.0 cm^2) to day 19 (1.5 cm^2). Progesterone reached maximum concentration on day 8 (12.8 ng/ml), and after that, the CL area and plasma progesterone decreased in parallel until the onset of luteolysis. During the luteolytic period, the decrease in plasma progesterone was about sixfold more significant than during the pre-luteolytic period. In contrast, the reduction in CL area and percentage of CL with the blood-flow area was approximately twofold greater (Ginther *et al*., 2007). The increase in the area of the granulosa layer from day 2 to day 1 before spontaneous ovulation is similar to the increase in the thickness of the granulosa layer from day 2 to day 1 that did not record any effect of age or age-day interaction during day 4 to day 1 before ovulation on dominant follicle diameter or the percent of follicular color blood flow (Ginther *et al*., 2009). In the current study, hCG, PG treatments, the mare's age, and the hot or cold seasons did not affect progesterone. In agreement with our results, serum progesterone on day 13 did not vary in mares subjected to ovulation induction using hCG during either breeding or non-breeding season (Teixeira *et al*., 2020).

The breeding of mares in our locality and the Middle East generally intensifies from October to May. It stops during the increased environmental temperature of the hot summer months, though the mare's cyclicity continues, and the photoperiod is at its maximum. This summer break of breeding may be recommended to minimize the effect of heat stress on the animal or to lower the quality of the green forage than in the cold

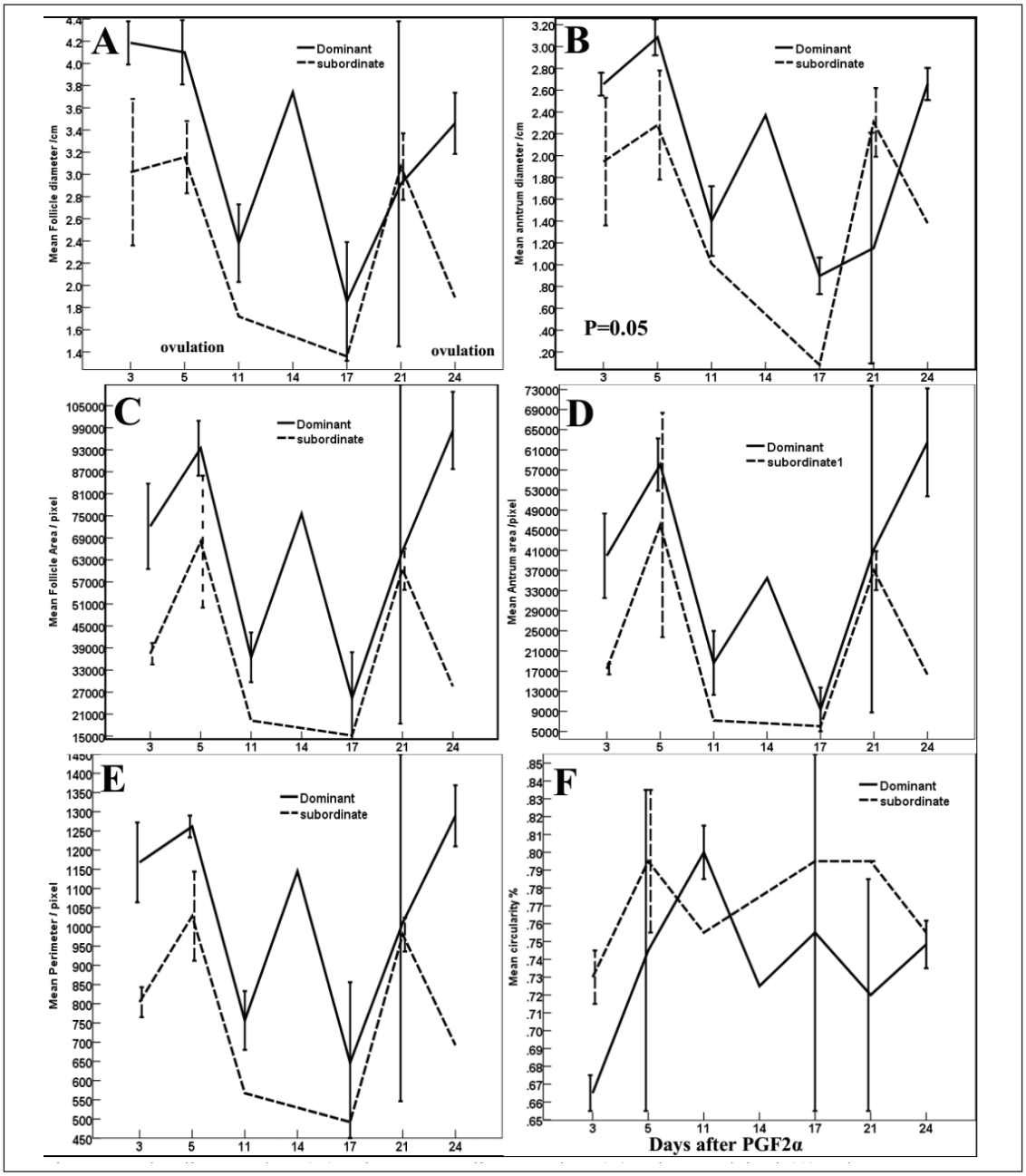

Fig. 6. Dominant and subordinate follicle values evaluated after inducing luteolysis using PGF2α in embryo donor mares. (A) Follicle diameter in cm. (B) Follicle antrum diameter in cm. (C) Follicle area in pixels, (D) follicle antrum area in pixels, (E) follicle perimeter in pixels, and (F) circulatory % with SEM bars.

months. The dominant follicles area, antrum area, perimeter, color area, and granulosa area increased in the hot months compared to the cold months. Though the environmental temperature during the summer months exceeded those of Brazilian environmental ones, the preovulatory dominant follicle diameter did not vary during months of high and low temperatures (Rua *et al*., 2019). The insignificant increased diameter of the dominant follicle during the hot summer months compared to the cold months of our study agrees with the slight increase in the diameter of the dominant follicle at day ten during summer compared to spring of the Brazilian tropical climate (Teixeira *et al*., 2020). In contrast to the significant increase in estradiol during the hot and cold seasons, no or slight increase

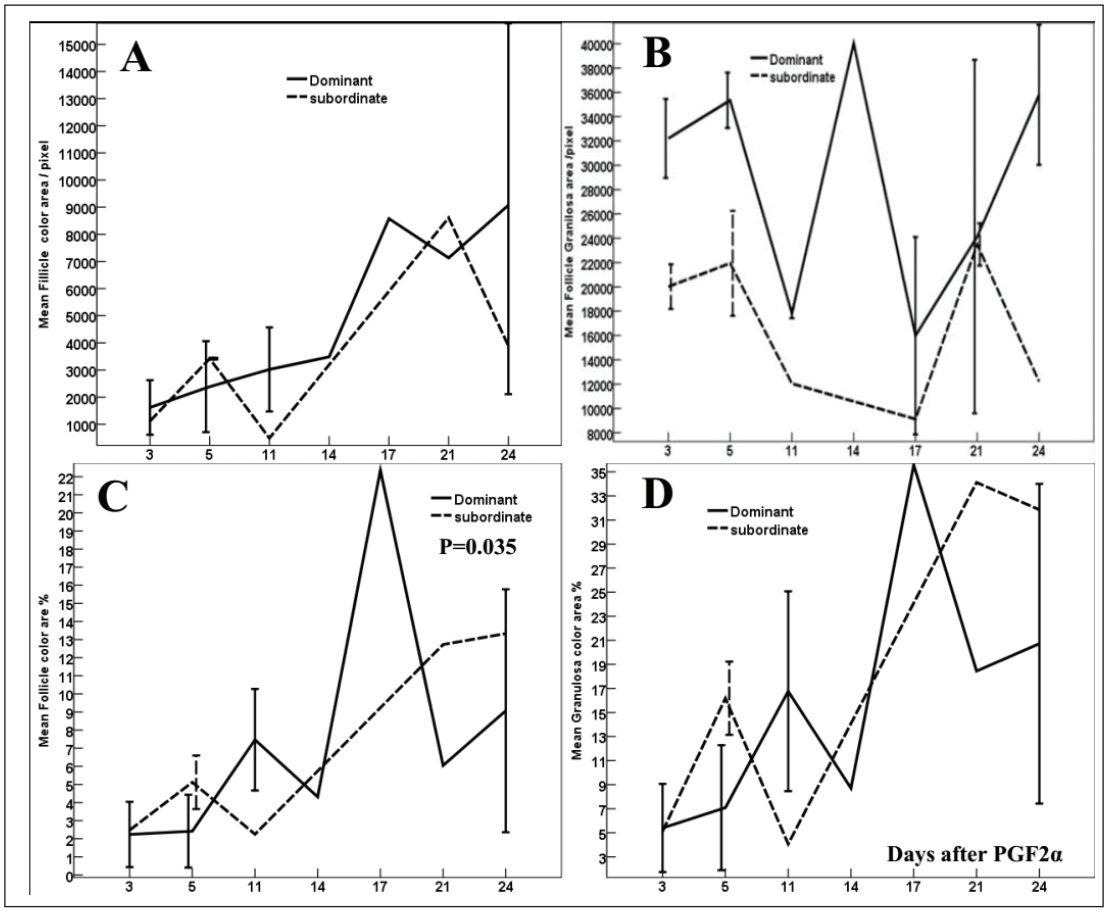

Fig. 7. Dominant and subordinate follicle values evaluated after inducing luteolysis using PGF2α in embryo donor mares. (A) Follicle color area in pixels, (B) follicle granulosa area in pixels, (C) follicle color area %, and (D) follicle granulosa color area %. Data are mean with SEM bars.

in estradiol was recorded in summer in Brazil (Teixeira *et al*., 2020).

Senile mares of the current study had dominant preovulatory follicles of nearly the same diameter as those of younger age (<10 years). Still, they had the lower area, antrum area, perimeter, and granulosa area but higher circulatory %, color area, and color area %. Similarly, thoroughbred mares kept in the Indian subtropical environments had nearly the number of mattings per oestrous, number of mattings per day 16 conception, the lowest percentages of day 16 pregnancies per estrus and season, and the highest percentages of early and late embryonic loss (Sharma *et al*., 2010). Similar to the insignificant difference in the maximum diameter of the dominant preovulatory follicle and at day 1 between mares >18 years and those <10 years, the diameter and the antrum diameter of the dominant follicle of the current study did not vary significantly (Ginther *et al*., 2009). Contrary to the decreased estradiol in senile mares, no change was recorded in estradiol (Ginther *et al*., 2009). The effect of age on the oocyte quality may refer to the e impaired transfer of carbohydrate and free fatty acid substrates from cumulus cells to the oocytes of old mares and the disruption of transzonal projections within the follicle between the cell types. Identifying age-associated alterations in the abundance of specific metabolites and their correlations among cells contributes to our understanding of follicular dysfunction with maternal aging (Catandi *et al*., 2023). In older mares, diet significantly affects the oocyte function by increasing the metabolic activity of oocytes, reducing their lipid content, and increasing their developmental potential (Catandi *et al*., 2022).

The increase of cholesterol and estradiol after inducing ovulation using hCG where the dominant follicle exceeded >30 mm diameter became mature and ovulated in 36 to 48 hours, associating the dominant follicle in the hot season and in younger mares similar to its increase of total cholesterol in follicular fluid of the large follicles indicating of increased the synthesis of steroids (Satué *et al*., 2019). LDH increased after hCG in our study's hot climate and younger mares. LDH is a marker of cell damage,

and its levels decrease as the follicle size increases (Satué *et al*., 2019).

Conclusion

During hot months, mares treated with hCG ovulated 24 hours later and prostaglandin-induced luteolysis was followed by new ovulation five days later. Follicles ovulated during the hot months were larger than those ovulated during the cold months and both had nearly the same color area %. Senile mares ovulated follicles with a lower area and antrum area but a higher color area %, so senile mares can be used as embryo or oocyte donors during the hot season. Neither hCG, PGF2α, age, nor hot climate influenced the dominant follicle diameters or progesterone. Hot climates and younger mares are exposed to more stress expressed by increased NO and LDH.

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Conflict of interest

The authors declare that they do not have any conflict of interest.

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Authors' contributions

Jamal M.H. Alkhadrawy performed the Doppler and Ultrasound scanning, funded the hormones, and performed the image analysis; Amal M. Aboelmaaty learned the ultrasound and Doppler examination, assayed the hormones, performed the statistical analysis, wrote the manuscript; Abdelraouf M. Ghallab put the experimental design and revised the submitted version of the manuscript; Mostafa M. Abou-Ahmed revised and approved the final version of the manuscript.

Data availability

All data supporting the findings of this study are available within the manuscript. Data from this article will be available upon request.

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