Veterinary Medical Journal – Giza
Faculty of Veterinary Medicine, Cairo University
(ISSN 1110 – 1423)
Accredited from National Authority for Quality Assurance and Accreditation
Giza, 12211 - Egypt

Ultrasonography And Hormonal Profile Of Hemorrhagic Anovulatory Follicles In Straight Egyptian Arabian Mares

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Abstract

The objective of the current study was to investigate the ultrasonographic characteristics and hormonal profile of mares affected with hemorrhagic anovulatory follicles (HAFs) in late follicular phase (3 days prior to the expected time of ovulation) to predict the development of the case. For this reason, normal cyclic Straight Egyptian Arabian mares (n=9) and those suffering from repeated hemorrhagic anovulatory follicle formation after 2 successive cycles (n=15) were investigated. Ultrasonographic ovarian examination was done using real time B-mode scanners. Ovarian examination and collection of blood samples were done daily during the late follicular phase till the occurrence of hemorrhage in affected mares and ovulation in control normal cyclic ones. Depending on the ultrasonographic examination, blood samples were selected at 3 days prior to expect time of ovulation (day-3) and on day of expected ovulation (day 0). The assaying hormones were Anti-Müllerian hormone (AMH), estradiol 17-β, progesterone and cortisol. Ultrasonographic characteristics did not show changes between the HAF and normal cyclic mares. Meanwhile, hormonal profile revealed significant decrease in the concentration of AMH in HAF group vs normal cyclic ones in day-3 and day 0. Unlike to this manner, estradiol concentration showed significant increase in HAF mares in the two periods of the study. Progesterone concentration recorded significant increase only in HAF affected mares on day 0 of study. Whereas, the cortisol concentration did not reveal any significant changes all over time of investigation. In conclusion, Ultrasonographic characteristics helped in the diagnosis of AHFs in mares rather than prediction. Whereas, lower concentrations of AMH could be served as predictor for the development of HAF in mares helping in appropriate management for this affection.

Keywords: Mares, Hemorrhagic anovulatory follicle, Ultrasonography, Hormonal profile.

Introduction

Anovulatory cycles in the mare represent a source of substantial reproductive inefficiency and economic loss to the equine breeding industry. When a mare fails to ovulate, these not only time and mare management costs lost, but valuable semen and the expense associated with its attainment, processing and handling may also be incurred as well **(Matcalf, 2010).** The mare is a seasonally polyestrus breeder characterized by cessation of cyclicity in the autumn as day light decreases. In the early spring, the mare inters "transition period" between the ovulatory season and the first ovulation of season which is characterized by a resurgence of follicular activity, irregular oestrous cycles and high incidence of regressing dominant follicles **(Ginther, 1992)**.

So, it is difficult to predict whether a follicle will ovulate or regress even for an experienced practitioner **(Cuervo-Arango and Clark, 2010).** Even with the use of ovulation induction drugs, anovulatory follicles not respond well **(Watson et al., 2004).**

In mares, blood extravasation into preovulatory follicles together with the failure of ovulation lead to development of Hemorrhagic Anovulatory Follicles (HAFs) which are mostly luteinized **(McCue and Squires 2002; Ginther et al., 2007; Cuervo-Arangao and Newcomb, 2010).** HAF formation occurred from a viable follicle and began on the day of expected ovulation; there was no indication that daily gray-scale ultrasonic imaging can be used to predict whether a follicle will ovulate or form HAF **(Ginther et al, 2007)**. In addition, there is no precursor signs of HAF formation and most of normal preovulatory characteristics are usually present **(Lefrance and Allen, 2003).** Ultrasonographic description of HAF is explained by **(Cuervo-Arango and Newcomb, 2009[a])** which is the previously fluid filled follicle of anechoic echotexture fills with echogenic specks that float freely in the follicular fluid and swirl if balloted, and without follicular collapse of the granulosa layer becomes increasingly echo dense and deeper. The number and echogenicity of the

ISSN1110-1423

intrafollicular specks increase but still have a mobile, swirling appearance. The follicle diameter increases and eventually the contents acquire a static organized appearance.

Hemorrhagic anovulatory follicles incidence has been linked to the age of the mare but also the use of hormonal treatments for induction of ovulation is involved **(Carnevle et al., 1989; Ginther et al., 1992; Cuervo-Arangao and Newcomb, 2008; Ginther at al., 2008).** The HAFs were developed in nontreaty and hCG treated mares **(Carnevle et al., 1989; Squine, 2002).** So, the exact cause of anovulatory follicles is still unknown and the mechanisms that generate different forms of anovulatory follicles may not be the same (**Metcalf and Roser, 2010**). Many researchers reported that this affection is repeatable, and the affected mares were called "Repeaters" **(Cuervo-Arango and Newcombe, 2009[a]). Ginther et al., (2006)** noticed mares during 13 estrous cycles and concluded that mares with no HAFs during the preceding season had none during 13 estrous cycles the next seasons. Controlled studies have shown in detail hormonal profile and Doppler ultrasonographic characteristics of development of HAFs in mares. **Ginther et al., (2007)** found only subtle differences in follicular wall vascularity between ovulatory and HAFs during 3 days prior to ovulation/ beginning of hemorrhage. In addition, hormonal profiles of LH, FSH and progesterone did not reveal any significant differences between mares with ovulatory versus anovulatory cycles, except for an elevation in the estradiol concentrations. The authors added that the follicular wall of anovulatory follicles was more vascularized one day prior to predicted ovulation.

Thus, prediction of the case would be the first step for prevention and treatment of the AHFs in mares. Additionally, it would be of great benefit to the horse breeding industry.

Anti-Müllerian Hormone (AMH), an ovarian specific product, is a glycoprotein a member of transforming growth factor β that plays an important role in the sex differentiation during embryogenesis. In male animal, testosterone is responsible for the development of Wolffian ducts, whereas AMH is needed to prevent the development of müllerian ducts into the uterus and other müllerian structures. In the female animals, the gonads do not produce AMH during sex differentiation in early gestation; so, the müllerain ducts automatically develop into normal female genitalia **(Ball et al., 2008).** Anti-Müllerian hormone is expressed by granulosa cells of the ovary controlling the formation of primary follicles by inhibiting excessive follicular recruitment by FSH **(Rico et al., 2009).** There were no significant changes in AMH concentrations during the estrous cycle or pregnancy **(Vanderwall and Rood.,2014).** In equines, AMH elevation indicates granulosa cell tumors (GCTs) **(Almeida et al., 2011; Ball et al., 2012; Gharagozlou et al., 2012).** Many researchers had dialed with its relationship with GCTs. Assessment of AMH level has recently been validated, as an additional diagnostic test for GCTs, the sensitivity of detection for AMH was 98% **(Ball et al., 2013).** Despite that, very little studies had dialed with its use as a diagnostic test for HAFs.

So far, information of systemic concentrations of hormones before and after formation of HAFs was spares. Hemorrhage may occur into a dominant follicle prior to ovulation resulting in HAF. Initially blood does not clot within the follicle due to the presence of a heparin like molecule within the follicular fluid which might account for the sequential changes seen on the ultrasonic images within structures and the variation among structures **(Ginther et al. 2007).** Anovulatory cycles in the mares represent a source of substantial reproductive inefficiency and economic loss to the equine breeding industry. So, development of predictive tool for HAFs would be of great benefit.

Therefore, the aim of the current study was to investigate the ultrasonographic characteristics and hormonal profile (anti-Müllerian Hormone, estradiol, progesterone and cortisol) of mares affected with hemorrhagic anovulatory follicles (HAFs) in late follicular phase (3 days prior to the expected time of ovulation) to predict the development of the case. To test these hypothesis HAFs repeaters mares were investigated in this study.

Materials and Methods

Experimental animals and housing

The current study was performed on 24 Straight Egyptian Arabians mares located in private stud farm, El-Haram, Giza, Egypt. Mares were 5-9 years old, with an average weight 400 kg. Mares were under ambient lighting before and during

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the study. They were kept in boxes with possibility of access into paddocks during the day. Mares were found suffering from repeated hemorrhagic anovulatory follicle formation after 2 successive cycles of hemorrhage formation. Mares were allocated into 2 groups:

Group (1): nine mares and serve as control normal cyclic.

Group (2): fifteen mares showed repeated anovulatory hemorrhagic follicle formation.

Nutrition

The animals were fed Egyptian clover (Trifolium alexandriunm) and legume hay. The animals were provided with concentrated ration and wheat straw in amounts sufficient to maintain body weight and had free access to water and mineralized salt.

Ultrasonographic ovarian examination:

Ultrasonography was performed using real time B-mode scanners **(Sonoscape -China)** equipped with 4-7.5 MHz frequency linear-array rectal transducer. The scanner has a built-in electronic caliper system for measuring distance, area and circumference, angle and auto follow measurements. Ultrasonic gel (Carboxymethylcellulose) was used as a lubricant during scanning. Ultrasonographic scan was performed once every day during estrous phase until the occurrence of hemorrhage formation and (in group under study) and till ovulation in control group.

Blood sampling and hormonal assay:

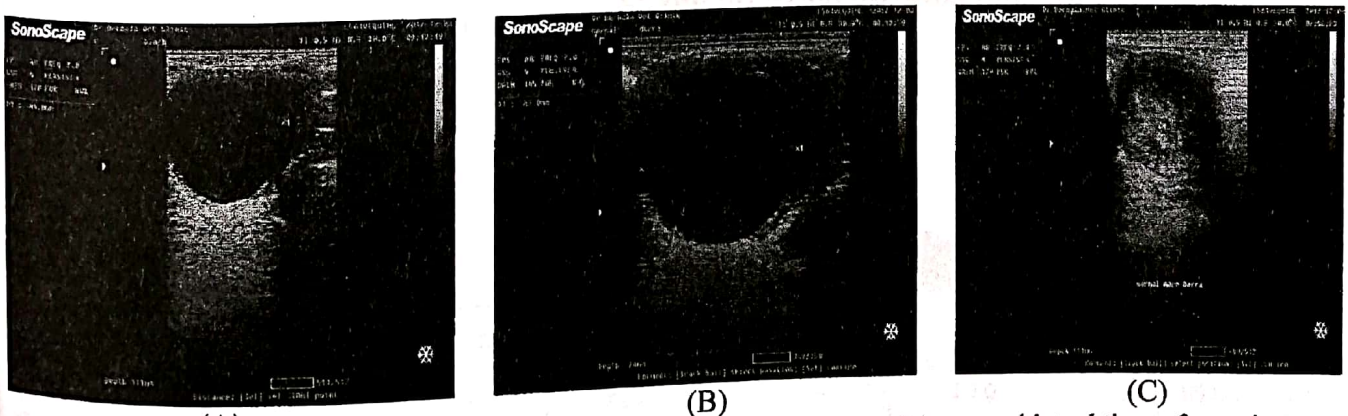
Blood samples were collected daily from each mare by jugular vein puncture in plain centrifuge tubes followed by centrifugation at 3000 rpm for 10 minutes to obtain serum then kept frozen at (-20° C) for hormonal assay. Samples for hormonal assay were selected depending on the ultrasonographic images at preovulatory and ovulatory stages.

Human AMH ELISA kit (Nova Tec, immunodiagnostica GmbH Waldstraβe Dietzenbach, Germany) was used to estimate serum AMH concentration. Human AMH was previously validated to equine AMH assay by **Gharagozlou et al., (2014)** and **Hyatt et al., (2015).**

The concentrations of estradiol 17-β and progesterone were assayed by ELISA technique using kits from Biochek, Inc. (CA.USA). Cortisol ELISA assay was done using diagnostic kit from Calbiotech USA.

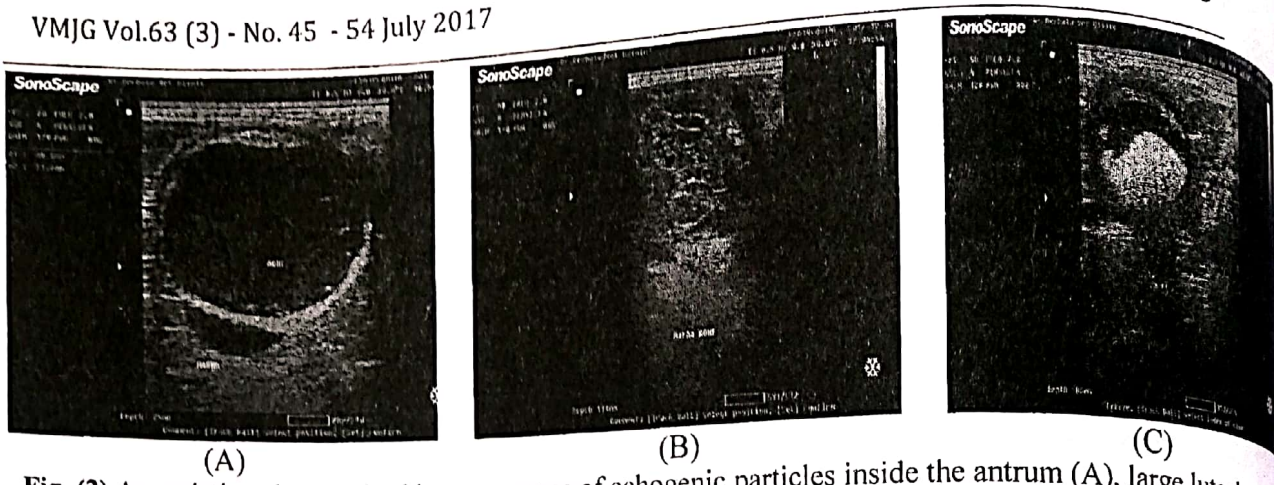
Results

Ultrasonographic scanning that was carried out in control normal cyclic mares during estrous phase showed normal growing follicles and appearance of normal mature follicle in the late follicular phase (Fig.1-A,B), Then followed by normal act of ovulation and formation of early CL (Fig.1-C) that appeared as echogenic grey granular structure. Meanwhile as in the affected mares there was normal growth of follicle but in the late follicular phase, the follicle enlarged and did not show normal act of ovulation but showed appearance of echogenic particles within the antrum of the follicles. In addition to appearance of jelly-like materials (Fig.2-A) that with time changed into large luteal tissue (Fig.2-B) or organized blood clot (Fig.2-C) that persisted for long period until the beginning of the next follicular phase.



(A) (B) (C)

Fig. (1) Normal growing follicle (A) mature follicle in late follicular phase (B), normal luteal tissue formation indicating normal ovulation (C).



(A) (B) (C)

Fig. (2) Anovulation characterized by appearance of echogenic particles inside the antrum (A), large luteal tissue formation (B), organized blood clot formation characterized by hyperechoic mass formation (C).

Regarding the results of hormonal profile, as shown in Fig (3) and (6) anti-Müllerian Hormone concentrations were significantly lower ($P<0.05$) in mares affected with HAFs in the two periods of the study (3 days before expected ovulation and day of expected ovulation, day -3 and day 0) compared with the control normal cyclic mares. As it recorded the concentrations of 1.64±0.05 ng/ml vs 2.44±0.10 ng/ml on day -3 and 1.69±0.06 ng/ml vs 2.48±0.05 ng/ml on day 0, respectively.

Oppositely, as shown in Fig (4) and (7) estradiol 17-β (E_2) concentrations were significantly higher ($P<0.05$) in mares affected with HAF compared with the normal cyclic mares in the two periods of investigation. As it recorded values of 2.96±0.17pg/ml vs 1.7±0.17pg/ml on day -3 and 4.99±0.29 pg/ml vs 3.38±0.21pg/ml on day 0. Also, Fig (6) showed that progesterone concentrations were significantly increased ($P<0.05$) on day 0 in HAF group as it recorded a concentration of 0.83±0.09 ng/ml vs 0.62±0.07 ng/ml in normal ovulatory group. Meanwhile, cortisol concentrations reveal no significant changes neither in HAF mares nor in normal cyclic ones all over the periods of study as shown in Fig (5 and 8). As it recorded a concentration of 56.58±1.62 ng/ml in AHF mares' vs 57.40±1.48 ng/ml in normal ovulatory ones on day -3. That concentrations did not differ on that of day 0 which recorded a concentration of 58.18±1.44 ng/ml in affected mares' vs 58.03±1.30 ng/ml in control ones.

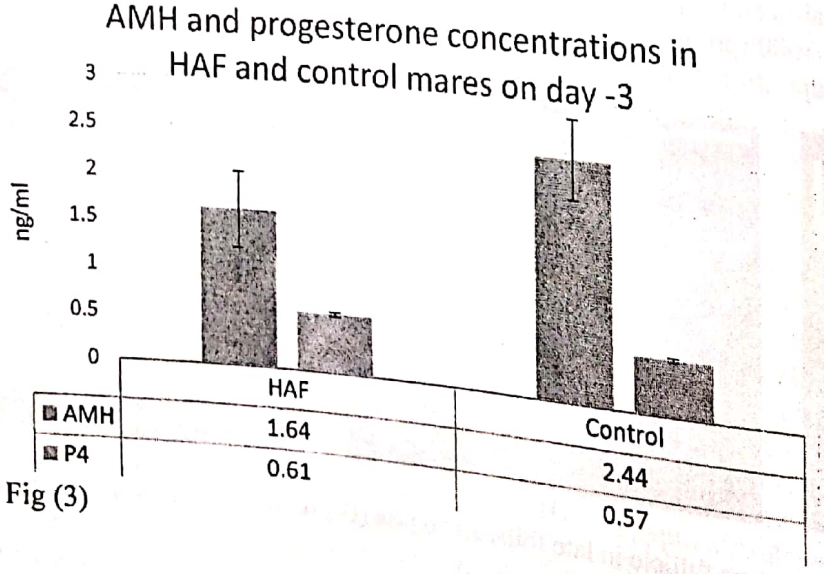


Fig (3)

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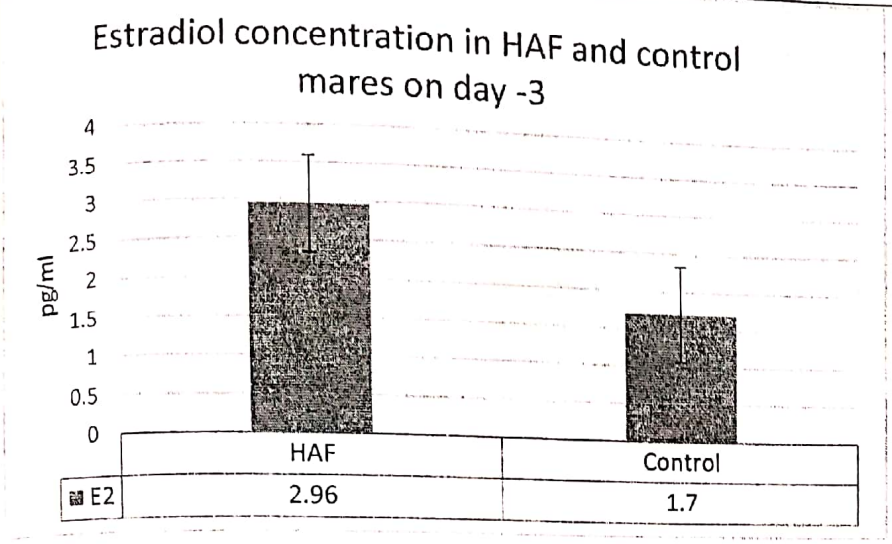

Fig (4)

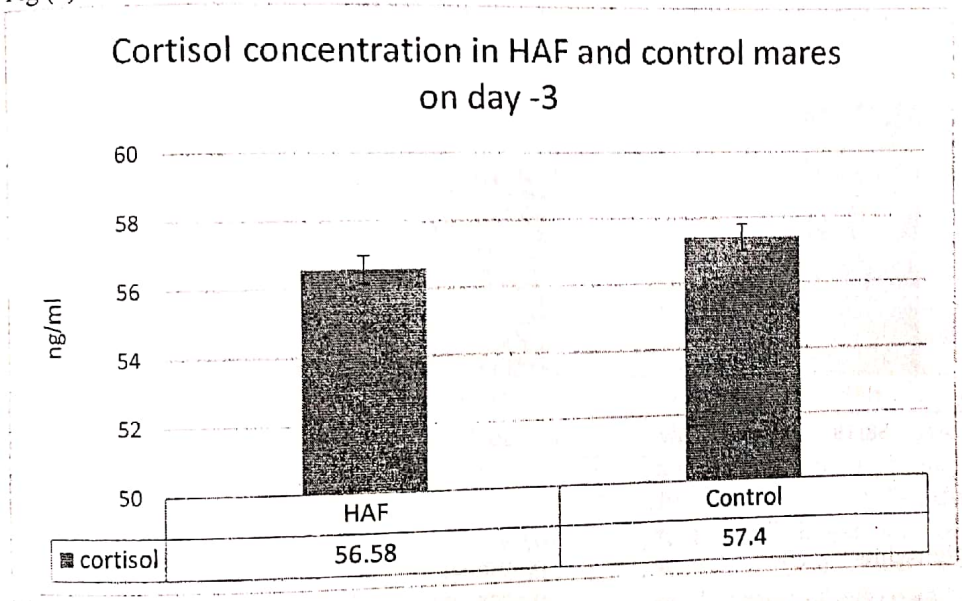

Fig (5)

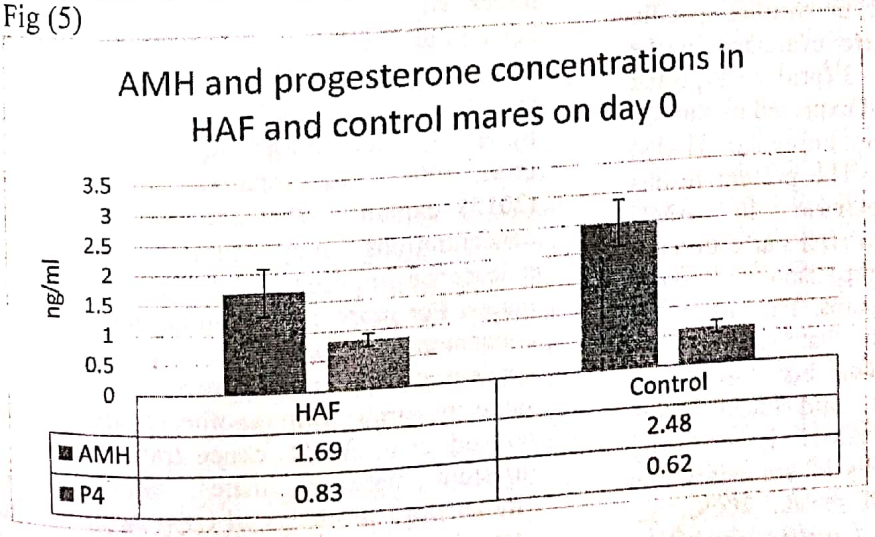

Fig (6)

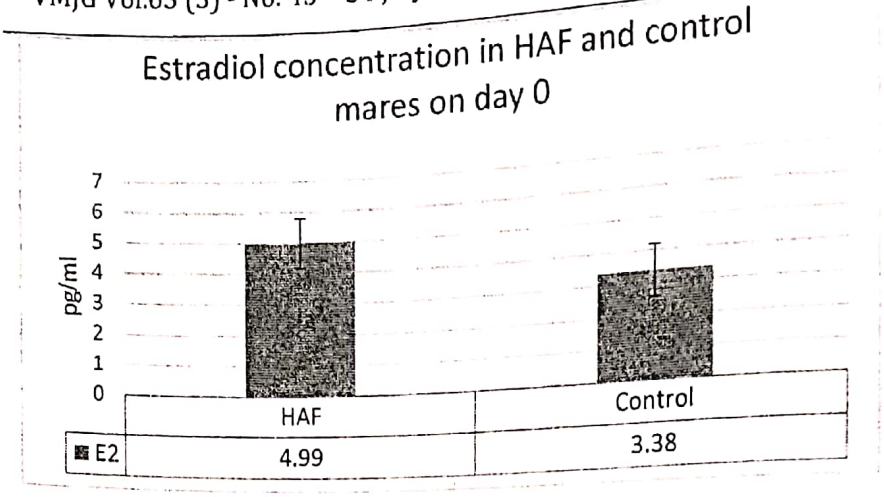

Fig (7)

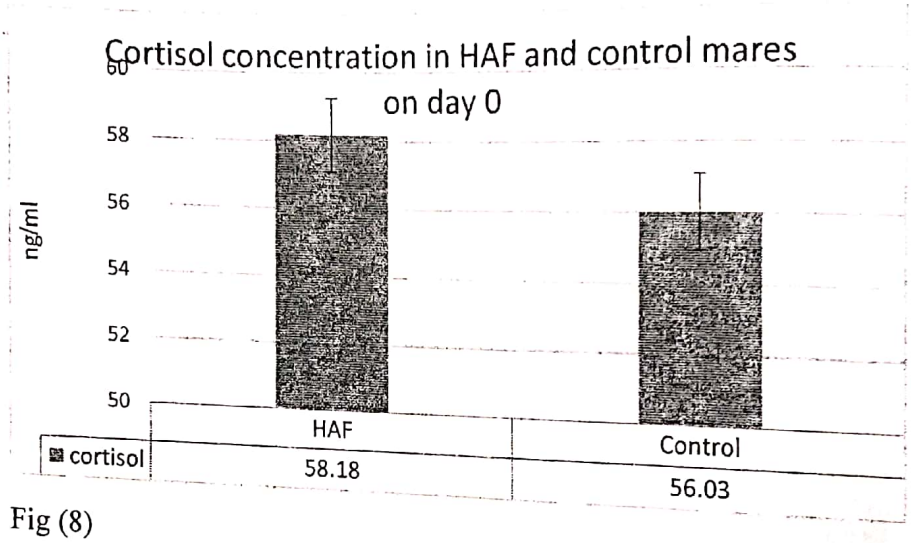

Fig (8)

Dicussion

In the current study, AMH, an ovarian specific product, concentrations were evaluated in the late follicular phase on day- 3 (prior to expected ovulation) and day 0 (day of expected ovulation) in Hemorrhagic Anovulatory Follicular (HAFs) and normal cyclic mares. The present results revealed significant decrease in AMH concentrations in mares with HAFs affection (on day -3 and day 0) in comparison with those periods in normal cyclic mares. This result was nearly in consistent with that recorded by **Gharagozlou et al., (2014),** but their blood samples were collected at mid luteal phase. Given that, in equine, AMH is primarily expressed by granulosa cells of secondary and small antral follicles **(Ball et al., 2008).** So, disruption in the cells function might be responsible for diminished follicular activity in mares with HAFs. Therefore, it seems that behavioral anestrous in that mares stems from the diminished granulosa cells function and corresponding reduced flocullogenesis imposed by HAFs **(McCue and Squires,2002; Ginther et al., 2007).** Additionally, **Gharagozlou et al. (2013)** explained that the decrease in AMH concentrations may indicate the ovarian quiescence imposed by anovulatory follicles in mares. For more confirmation, the serum AMH concentrations increased to its normal concentration after disappearance of HAFs. The same researchers in another study at (2014) noticed that AMH concentrations were not different between mares with Luteinized Anovulatory Follicles (LUFs) with ovulatory ones illustrating that LUFs do not impose the diminished granulosa cells function as do HAFs.

ISSN1110-1423

Also, **Guervo-Arango and Newcombe (2012)** concluded that these two types of anovulatory follicles (HAFs and LUFs) appear to share similar ultrasound features. They added that none of the following endpoints differ significantly between them, follicular diameter, follicular contents score, interval form hCG administration to beginning of follicular hemorrhage and interval from hemorrhage to organization of follicular contents. Many researchers previously had dialed with Granulosa Cell Tumors (GCTs) and recorded a great elevation in AMH concentrations **(Ball et al., 2013; Hyatt et al., 2015 and Murase et al., 2018;** in press) which may reach five folds of its normal concentration as recorded by **Vanderwall and Rood (2014).** Moreover, **Ginther at el., (2007)** showed that mares with preovulatory follicles show the same ultrasonographic follicular wall characteristics, uterine edema patterns and reproductive hormonal profiles during the 3 days prior to ovulation/hemorrhage. Therefore, AMH concentrations could be used as a diagnostic tool to differentiate between some cases of preovulatory affections (HAFs, LUFs and GCTs) especially when the ultrasonographic images do not give sharp demarcation between them.

Unlike AMH, estradiol 17-β showed the opposite manner of changes as its concentration were increased significantly in HAFs mares in comparison with normal cyclic ones in the two periods of study. This result was in accordance with that recorded previously by **Ginther et al. (2007 and 2008)** as they dialed with HAFs mares and normal cyclic ones in late follicular phase and reported that in both groups, the preovulatory estradiol surge reached a peak 2 days before ovulation or anovulation. They added that estradiol was higher in HAF mares on day -3 and this difference was confirmed when largest follicle was a mean of 32 mm. The role elevated estradiol in the formation of HAFs a few days later is not known but greater vascularization of the HAF could be involved **(Ginther et al., 2006).** In **2008,** the same authors in another study recorded that there was a close temporal and mechanistic relationship occur between estradiol and FSH and between estradiol and LH as it has a negative effect on both FSH and LH. When estradiol decreases, the negative effect diminishes and accounts for the beginning of an FSH increase and a transition from a slow to rapid increase in LH on the day of the estradiol peak. The decrease in estradiol and the reduction or cessation in the growth of the preovulatory follicle beginning 2 days before ovulation are attributable to the development of a reciprocal negative effect of LH on follicle estradiol production when LH reaches a critical concentration.

With respect of progesterone concentrations, the current study showed a significant increase in its concentration in HAF mares on day 0 of the study. This result agrees with **McCue and Squires (2002) Cuervo-Arangao and Clark (2010)** who stated that HAF mares are mostly luteinized and the luteal tissue was associated with production of progesterone during HAF development. Moreover, **Ginther et al. (2008)** recorded a close negative relationship between progesterone and LH. According to the last explanations, we can explain the significant increase in progesterone concentrations on day0 which could be effect on the LH surge required for successful ovulation. In earlier studies, **Ginther (1992)** based on gross appearance during transection and stated that luteinization is consistent with the report that the wall of HAF consisted of 2-5 mm thickness. The luteinized wall was well vascularized, and the vascularization remained extensive as the structure regressed. **Ginther et al. (2007)** found that the follicular wall of anovulatory follicles was more vascularized 1 day prior to predicted ovulation in comparison to follicles undergoing successful ovulation. In another study, **Ellenberger et al. (2009)** ovarian cell populations were examined with immunohistochemistry for expression of Vascular Endothelial Factors. They concluded that, despite the apparent expression of sufficient VEGF-A in the AHFs to allow ovulation and corpus luteum formation, there was a lack of the receptor, FIK-1, which affects the pro-angiogenic activity of VEGF-A, which could be a reason for ovulation failure associated with HAF formation.

Although not studied critically, negative correlation seems to be present between AMH and progesterone concentrations in the studied HAF affected mares which was previously

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demonstrated in cattle (**Monniaux et al., 2008**) and in humans (**Fanchin et al., 2007**). Regarding the cortisol concentrations, the present work did not reveal any significant changes in the two groups of investigation during the two periods of the study. **Metcalf and Roser (2010)** concluded that preovulatory cortisol concentration was found to be not significantly different between mares with normal ovulatory cycles and those with anovulatory ones. Meanwhile, **Asa et al. (1982)** stated that cortisol is associated with follicular development and ovulation in mares. Furthermore, **Johnson et al. (2004)** stated that the case may be accompanied with laminitis and concluded that the protracted laminitis and the associated stress often results in increase in systemic cortisol. Herein, none of the affected mares were lame during the time blood samples collected. Therefore, the cortisol hypothesis was not adequate tested.

Using ultrasonography scanning for normal and affected mares only showed the formation of HAF through presence of echogenic particles inside the antrum, as well as jelly-like material that changed into large luteal tissue but could not predict pre-hemorrhage and anovulation as the characters of the preovulatory follicle in control and HAF affected mares were the same. Only in some mares when remaining organized blood clot formed in previous anovulatory cycle can be a predictor for next anovulation probability. These results were quite similar to those obtained by **Ginther el al, (2007), Lefrance and Allen (2003), Cuervo-Arango and Newcomb (2009[b]).** Consequently, ultrasonography is more accurate in diagnosis rather than prediction.

Conclusion

The current study has dialed with HAFs Straight Egyptian Arabians mares in late follicular phase prior to expected time of ovulation in an attempt to predict the case through ultrasonographic characteristics and hormonal profiles. Ultrasonographic characteristics revealed no differences in criteria between preovulatory follicles in normal ovulatory cycle and in HAF before anovulation. Whereas, most characteristic feature was the significant decrease in the concentration of AMH associated with increase in the concentration of estradiol 3 days prior to expected time of ovulation. The repeatability of AMH assay could make it a valuable practical method for prediction and differentiation of HAFs from other ovarian affections in mares.

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الملخص العربى

دراسة بالموجات فوق الصوتية و الصورة الهرمونية للمهرات العربية الأصيلة التى تعانى من الجريبات النزيفية اللاباضية

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تهدف هذه الدراسة الى التنبؤ بحدوث حالات الجريبات النزيفية اللا اباضية فى المهرات العربية الأصيلة فى مصر. وذلك من خلال فحص المبيض بالموجات فوق الصوتية و الصورة الهرمونية للمهرات المصابة. وقد اجريت الدراسة على 24 مهرة تم تقسيمها الى مجموعتين. الاولى تشمل 15 مهرة سبق لها وأن عانت من هذه الحالة دورتين شبق سابقتين على الأقل. والمجموعة الثانية وتشمل 9 مهرات ذات دورات شبق منتظمة وتبويض منتظم وقد كانت المجموعة الضابطة. تم فحص مبايض جميع المهرات بالموجات فوق الصوتية يوميا لمدة سبع ايام فى مرحلة ماقبل الشياع وقبل التوقيت المتوقع للتبويض. كما تم تجميع عينات دم من المهرات فى نفس التوقيت وفصله للحصول على السيرم وحفظه مجمدا لحين استخدامه فى قياس تركيز الهرمونات. وبناء على نتائج الفحص بالموجات فوق الصوتية تم اختيار عينات الدم والتى كانت فى اليوم الثالث قبل التوقيت المتوقع للتبويض فى المجموعتين وفى يوم التبويض الذى تم بالفعل فى المجموعة الضابطة بينما فشل ولم يتم فى المجموعة المصابة وتحولت الجريبات الى جريبات نزيفية ولم يتم التبويض. وكانت الهرمونات المعنية بالفحص هى هرمون الانتى موليريان والاستروجين والبروجستيرون والكورتيزول. وكانت نتائج الدراسة كالتالى: بالنسبة للفحص بالموجات فوق الصوتية فقد تم عن طريقه تشخيص الجريبات النزيفية فى اليوم المتوقع للتبويض ولم تظهرفروق معنوية قبل ذلك. فيما أظهرت نتائج الفحص الهرمونى نقصا معنويا فى تركيز هرمون الأنتى موليريان فى مجموعة الجريبات النزيفية مقارنة بالمجموعة الضابطة فى اليوم الثالث قبل التبويض ويوم التبويض المتوقع نفسه. وعلى العكس من هذا سجل هرمون الاستروجين زيادة معنوية فى فترتى الدراسة فى مجموعة الجريبات النزيفية. بينما أظهر هرمون البروجستيرون زيادة معنوية فى مجموعة الجريبات النزيفية فى اليوم المتوقع لحدوث التبويض. فيما لم يسجل هرمون الكورتيزول اى تغير معنوى بين المجموعتين خلال فترتى التجربة. ونستخلص من هذه الدراسة ان متابعة المهرات بالموجات فوق الصوتية فى فترة ماقبل التبويض يساعد على تشخيص حالات الجريبات النزيفية بينما يمكن التنبؤ بحدوث الجريبات النزيفية من خلال نقص تركيز هرمون الأنتى موليريان مما يعطى فرصة اكبر للتعامل مع الحالة قبل تطورها وما يتبع ذلك من أضرار للحيوان وخسائر اقتصادية للمربين.