Title	Hormonal composition of follicular fluid from abnormal follicular structures in mares
Authors(s)	Beltman, Marijke Eileen, Walsh, S. W., Canty, Mary J., Duffy, P., Crowe, Mark
Publication date	2014-10
Publication information	Beltman, Marijke Eileen, S. W. Walsh, Mary J. Canty, P. Duffy, and Mark Crowe. "Hormonal Composition of Follicular Fluid from Abnormal Follicular Structures in Mares." Elsevier, October 2014. https://doi.org/10.1016/j.rvsc.2014.10.007.
Publisher	Elsevier
Item record/more information	http://hdl.handle.net/10197/6118
Publisher's statement	This is the author's version of a work that was accepted for publication in Research in Veterinary Science. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in Research in Veterinary Science (VOL 97, ISSUE 3, (2014)) DOI: 10.1016/j.rvsc.2014.10.007
Publisher's version (DOI)	10.1016/j.rvsc.2014.10.007

Short communication

Hormonal composition of follicular fluid from abnormal follicular structures in mares.

Beltman M.E.[1*], Walsh S.W.[1], Canty, M.J.[2], Duffy P.[1], Crowe M.A[1].

1. *UCD School Veterinary Medicine, University College Dublin, Ireland*
2. *Department of Agriculture, Food and the Marine, Backweston Campus, Youngs Cross Celbridge, Co. Kildare, Ireland*

*****Corresponding Author: Phone: +353-1-7166084, Fax: +353-1-7166005

Email: marijke.beltman@ucd.ie

Abstract

The objective was to characterise the hormonal composition of follicular fluid from mares with distinct anovulatory-cystic follicles. Follicular fluid was aspirated from six mares that presented with cystic follicles and from pre-ovulatory follicles of five normal mares (controls). Differences in progesterone, oestradiol, testosterone, IGF-I and IGF binding were analysed using Fisher's exact test. There were greater ($P<0.03$) follicular fluid oestradiol concentrations in normal follicles and the testosterone concentration of the cystic fluid was greater ($P<0.05$) than that of the normal fluid. There also was a greater ($P<0.03$) percentage of IGF-I binding and lower ($P<0.02$) IGF-I concentrations in the fluid collected from the cystic structures compared with the fluid from normal follicles. Despite the limited number of animals, the fact that fluid aspirated from cystic follicles had higher testosterone and lower oestradiol concentrations could be of diagnostic value when a practitioner wants to distinguish between a cystic and non-cystic persistent follicle. The research reported here also indicates a likely role for the IGF system in the pathogenesis of the development and maintenance of anovulatory follicular structures in mare ovaries.

Key words: Mare, abnormal follicles, IGF, pathogenesis, oestradiol, progesterone.

Infertility in mares contributes to losses in the equine industry and can have multiple origins, one of which is ovarian, with most pathologies being of follicular origin and the most common conditions found are granulosa cell tumours (GCT's), haemorrhagic follicles and cystic follicles (McCue, 1998; Ginther *et al.*, 2007). Follicular growth during the cycle is tightly regulated by a number of locally as well as centrally produced hormones. Selection of the future dominant follicle occurs due to a combination of two main processes: the development of receptors for LH in its granulosa cells together with the increase of free IGF-I (Beg and Ginther, 2006). Free IGF-I is available due to the influence of PAPP-A which degrades insulin-like growth factor binding proteins (IGFBP) 4 and 5 within the follicular fluid (Monget *et al.*, 2002; Fortune *et al.*, 2004; Spicer, 2004), leading to a decrease in IGFBP concentration when the follicle grows (Canty *et al.*, 2006). The activities of IGFBP-2, -4 and -5 however, are less in growing dominant follicles compared with subordinate follicles (Gerard *et al.*, 2004) and are therefore suggested to be closely related to the physiological state of follicles (Gerard and Monget, 1998)

Haemorrhagic follicles are the result of ovulation failure of endocrine origin (Ginther *et al.*, 2007) and are the result of haemorrhage into the follicle which is followed by re-organization of follicular contents and in most cases luteinisation of the follicular wall (Ginther *et al.*, 2007; Cuervo-Arango and Newcombe, 2012). This condition, which can persist for up to two months, can occur during the spring and autumn transition period as well as during the normal breeding season and is also reported after hormonal treatment with cloprostenol to short cycle mares (Cuervo-Arango and Newcombe, 2009; 2010). The majority of haemorrhagic follicles will regress spontaneously over a period of one to four weeks (McCue, 1998).

Cystic follicles in mares have not been described as extensively as in ruminants but still occur, although there is on-going discussion about their actual definition. Sometimes these follicles are referred to as persistent anovulatory follicles (McCue, 1998), which can lead to confusion with a haemorrhagic follicle. Either way, the pathophysiology of a large follicle that grows and does not ovulate remains unclear; although the assumption is that the follicles persist and grow after ovulation failure similar to ruminants. The aim was to characterise the composition of follicular fluid from mares with distinct anovulatory follicles of cystic proportions and compare this with follicular fluid collected from normal mares. By doing this we hoped to gain some more insight into the pathophysiology behind this presentation of abnormal ovarian structures. The hypothesis was that follicular fluid of these abnormal follicles would have different concentrations of oestradiol, IGF-I/IGFBP and testosterone when compared with that of mares with normal follicles. We also hypothesised that there would be a role for the IGF system in the pathogenesis behind the development of this type of anovulatory (cystic) follicles in mares.

All procedures involving normal animals were approved by the University's Animal Research Ethics Committee (AREC-P-07-43) and were carried out under licence from the Department of Health (Cruelty to Animals Act, Ireland, 1876, as amended by EU directive 86/609/EC). Six mares, all Irish sport horses ranging in age from 4-17 years presented with a history of having one ovary with a follicle that did not respond to any exogenous hormone treatment and that interfered with normal cyclicity. All mares were kept in commercial grass based management systems. None of the follicles fitted the description of a haemorrhagic follicle on ultrasound and thus were defined as cystic. All follicles were aspirated using a transvaginal ovum pick up (OPU) procedure as previously described by (Mari *et al.*, 2005). Following collection, the follicular fluid was centrifuged to remove the granulosa cells after which it was frozen and stored at -80 ºC. Follicular fluid from pre-ovulatory follicles from five normal mares was also collected to enable analyses and comparison with that from the cystic follicles. This was done once they reached a size of 40 mm following synchronisation with 0.5 ml of a PGF2α analogue (125 μg of cloprostenol; Estrumate, Intervet, Dublin, Ireland). Each mare was scanned transrectally (7.5MHz linear probe) to determine the status of her follicular development and to determine when and which follicles could be aspirated. After this was determined, follicular fluid was aspirated as described above. All mares recovered well post aspiration, resumed normal cyclicity and were able to conceive during subsequent matings.

After diluting the follicular fluid to 1:100, progesterone, oestradiol and testosterone concentrations were measured using validated radioimmunoassay (RIA) procedures as previously described (Prendiville *et al.*, 1995; Forde *et al.*, 2010; García-Herreros *et al.*, 2010). Assay sensitivities were 0.2 pg/ml (oestradiol), 0.03 ng/ml (progesterone) and 2 ng/ml (testosterone). Intra-assay coefficients of variation (CV) ranged between 1.0 and 7.8 % for low, medium, and high reference samples in all three assays, all samples were analysed within a single assay for each analyte. IGF-I concentrations in follicular fluid were determined using a radioimmunoassay following which followed ethanol-acetone-acetic acid extraction (at a ratio of > 60:30:10, as described previously by Beltman *et al.,* 2010) with recombinant iodinated IGF-I (Upstate, Millipore, Temecula, CA, USA) as the standard and 50 μl anti-human IGF-I (NHPP-NIDDK AFP 4892898; National Hormone and Peptide Program, Torrance, CA, USA; dilution 1:750,000) as the primary antibody. The sensitivity of the assay was 6 pg per tube (6 ng/ml). Follicular fluid IGF-I was run in one assay and the intra-assay CV was 10.3% for samples containing a mean concentration of 149.7 ng/ml. Total IGFBP activity in follicular fluid was determined following incubation with ^{I25}I-labelled IGF-I by the method described by (Simpson *et al.*, 1994). Briefly, 50 μl aliquots of follicular fluid were incubated overnight at 4°C with 100 μl ^{I25}I-labelled IGF-I (1.9×10^{6} cpm/100 μl) and 150 μl of assay buffer (PBS containing 2.5 mg BSA ml^{-1}, pH 7.5). Activated charcoal (500 μ1;

5% w/v in PBS containing 2.5 mg BSA/ml) was added to each tube to separate bound from free ^{I25}I-labelled IGF-I. The tubes were then incubated for 30 min at 4°C, and centrifuged at 2000 rpm for 20 min at 4°C. The supernatant was counted using a gamma counter (Wizard 1470; Wallac/Perkin Elmer, Turku, Finland).

Differences in progesterone, oestradiol, testosterone, IGF-I and IGF binding were analysed by using a Fisher’s exact test as described by (Snedecor and Cochran, 1989) using SPSS for Windows.

The average size of the follicles aspirated from the normal mares was 42.5±1.2 mm with the average size of the cystic follicles measuring 76.7±6.11mm ($P<0.01$). Follicular fluid pogesterone, testosterone, oestradiol, IGF-I and IGFBP binding percentage data are presented in table 1. Follicular fluid oestradiol concentrations were lower ($P<0.03$) in the cystic follicles than in the normal pre-ovulatory follicles. The testosterone concentration in the follicular fluid from cystic follicles was greater than ($P<0.05$) that in normal fluid. There was a greater ($P<0.03$) percentage of IGF-I binding in the fluid collected from the cystic structures than in normal follicles. The IGF-I concentrations were lower ($P<0.02$) in the cystic structures when compared with the normal follicles.

True follicular cysts that are persistent and anovulatory are relatively uncommon in mares with little evidence that they remain hormonally active to interfere with subsequent cyclicity and fertility (McCue, 1998). However, in all the mares that we describe here, the persistent follicular structure on one of the ovaries did interfere with normal cyclicity, resulting in abnormal cyclical patterns and anoestrus. As such we believe that these follicular structures were truly active cysts, which is also supported by the clinical finding that the mares resumed normal cyclicity as well as fertility after the aspiration of the abnormal follicle.

In mares, the concentration of free IGF-I is higher in large follicles in the follicular phase when compared with the follicles from the luteal phase. This increased concentration was also associated with an increase in oestradiol:progesterone ratio in the follicular fluid (Spicer *et al.*, 2005).

We found a larger percentage binding of IGF-I in the fluid that was aspirated from the cystic structures when compared with the fluid that was aspirated from the normal mares, together with lower concentrations of total IGF-I in the fluid. This would indicate that there was a larger concentration of IGFBP's present in this fluid, indicating that there could be a similar role for the IGF system in the development of these follicles as has been shown in cattle (Rodríguez *et al.*, 2011). The lower concentrations of oestradiol that we found can be explained by the fact that the free IGF-I in follicular fluid works synergistically with FSH to enhance oestradiol synthesis within the follicle (Fortune *et al.*, 2004). With the presence of low concentrations of IGF-I it is therefore not surprising that we found lower concentrations of oestradiol in the cystic follicles.

The fact that we found higher concentrations of testosterone in the fluid from the cystic structures than in the normal structures indicates that the theca cells in these follicles are producing more of this steroid hormone, suggesting that there is either LH involvement in the growth and maintenance of the cystic structures (Santiago *et al.*, 2005; Vanholder *et al.*, 2006) or that there is less aromatase available to convert the testosterone to oestradiol (Bosh *et al.*, 2009).

In conclusion, despite the fact that we only had six mares with an abnormal follicle, there were significant differences between the cystic and normal follicles. The pathogenesis of cystic follicles in mares appears to be very similar to what is known about the pathophysiology of follicular cysts in cattle, with the IGF system playing a role in both conditions causing the differences in testosterone and oestradiol concentrations that were found Fluid aspirated from cystic follicles had higher testosterone and lower oestradiol concentrations when compared with follicular fluid from normal follicles which could be of diagnostic value when a practitioner wants to distinguish between a cystic and non-cystic persistent follicle. However, reasons for the de-regulation in the IGF system in mares leading to this condition remain unclear and should be explored further with more cases.

Acknowledgements

The authors thank the horse owners and staff at UCD Veterinary Hospital for access to patients. Furthermore, they thank Dr. Vivian Gath for his technical assistance with follicle aspiration, Penny Furney and Sheila Ni Cheallaigh for help with hormone assays and the staff of UCD Lyons Research Farm for access to normal mares. They also thank Dr. L.J. Spicer for assistance with the IGF-1 radioimmunoassay and Dr. Parlow the National Hormone & Peptide Program (NHPP) for supplying the anti-hIGF-1, NHPP-NIDDK.

References

Beg, M.A., Ginther, O.J., 2006. Follicle selection in cattle and horses: role of intrafollicular factors. Reproduction 132, 365-377.

Beltman, M.E., Forde, N., Furney, P., Carter, F., Roche, J.F., Lonergan, P., Crowe, M.A., 2010. Characterisation of endometrial gene expression and metabolic parameters in beef heifers yielding viable or non-viable embryos on Day 7 after insemination. Reproduction, fertility and development 22, 987-999.

Bosh, K.A., Powell, D., Shelton, B., Zent, W., 2009. Reproductive performance measures among Thoroughbred mares in central Kentucky, during the 2004 mating season. Equine Veterinary Journal 41, 883-888.

Canty, M.J., Boland, M.P., Evans, A.C.O., Crowe, M.A., 2006. Alterations in follicular IGFBP mRNA expression and follicular fluid IGFBP concentrations during the first follicle wave in beef heifers. Animal Reproduction Science 93, 199-217.

Cuervo-Arango, J., Newcombe, J.R., 2009. The effect of hormone treatments (hCG and cloprostenol) and season on the incidence of hemorrhagic anovulatory follicles in the mare: A field study. Theriogenology 72, 1262-1267.

Cuervo-Arango, J., Newcombe, J.R., 2010. Risk Factors for the Development of Haemorrhagic Anovulatory Follicles in the Mare. Reproduction in domestic animals 45, 473-480.

Cuervo-Arango, J., Newcombe, J.R., 2012. Ultrasound characteristics of experimentally induced luteinized unruptured follicles (LUF) and naturally occurring hemorrhagic anovulatory follicles (HAF) in the mare. Theriogenology 77, 514-524.

Forde, N., Beltman, M.E., Duffy, G.B., Duffy, P., Mehta, J.P., O'Gaora, P., Roche, J.F., Lonergan, P., Crowe, M.A., 2010. Changes in the endometrial transcriptome during the bovine estrous cycle: Effect of low circulating progesterone and consequences for conceptus elongation Biology of Reproduction.

Fortune, J.E., Rivera, G.M., Yang, M.Y., 2004. Follicular development: the role of the follicular microenvironment in selection of the dominant follicle. Animal reproduction science 82-83, 109-126.

García-Herreros, M., Bermejo-Álvarez, P., Rizos, D., Gutiérrez-Adán, A., Fahey, A.G., Lonergan, P., 2010. Intrafollicular testosterone concentration and sex ratio in individually cultured bovine embryos. Reproduction, fertility and development 22, 533-538.

Gerard, N., Delpuech, T., Oxvig, C., Overgaard, M.T., Monget, P., 2004. Proteolytic degradation of IGF-binding protein (IGFBP)-2 in equine ovarian follicles: involvement of pregnancy-associated plasma protein-A (PAPP-A) and association with dominant but not subordinated follicles. Journal of Endocrinology 182, 457-466.

Gerard, N., Monget, P., 1998. Intrafollicular insulin-like growth factor-binding protein levels in equine ovarian follicles during preovulatory maturation and regression. Biology of Reproduction 58, 1508-1514.

Ginther, O.J., Gastal, E.L., Gastal, M.O., Beg, M.A., 2007. Incidence, Endocrinology, Vascularity, and Morphology of Hemorrhagic Anovulatory Follicles in Mares. Journal of Equine Veterinary Science 27, 130-139.

Mari, G., Barbara, M., Eleonora, I., Stefano, B., 2005. Fertility in the mare after repeated transvaginal ultrasound-guided aspirations. Animal Reproduction Science 88, 299-308.

McCue, P.M., 1998. Review of Ovarian Abnormalities in the Mare, Proceedings of the Annual Convention of the American Association of Equine Practitioners.

Monget, P., Fabre, S., Mulsant, P., Lecerf, F., Elsen, J.-M., Mazerbourg, S., Pisselet, C., Monniaux, D., 2002. Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. Domestic Animal Endocrinology 23, 139-154.

Prendiville, D.J., Enright, W.J., Crowe, M.A., Finnerty, M., Hynes, N., Roche, J.F., 1995. Immunization of heifers against gonadotropin-releasing hormone: antibody titers, ovarian function, body growth, and carcass characteristics. J. Anim Sci. 73, 2382-2389.

Rodríguez, F.M., Salvetti, N.R., Panzani, C.G., Barbeito, C.G., Ortega, H.H., Rey, F., 2011. Influence of insulin-like growth factor-binding proteins-2 and -3 in the pathogenesis of cystic ovarian disease in cattle. Animal reproduction science 128, 1-10.

Santiago, C.A., Voge, J.L., Aad, P.Y., Allen, D.T., Stein, D.R., Malayer, J.R., Spicer, L.J., 2005. Pregnancy-associated plasma protein-A and insulin-like growth factor binding protein mRNAs in granulosa cells of dominant and subordinate follicles of preovulatory cattle. Domestic Animal Endocrinology 28, 46-63.

Simpson, R.B., Chase, C.C., Spicer, L.J., Vernon, R.K., Hammond, A.C., Rae, D.O., 1994. Effect of exogenous insulin on plasma and follicular insulin-like growth factor I, insulin-like growth factor binding protein activity, follicular oestradiol and progesterone, and follicular growth in superovulated Angus and Brahman cows. Journal of Reproduction and Fertility 102, 483-492.

Snedecor, G.W., Cochran, W.G., 1989. Statistical Methods. Ames Iwoa State University Press, Iowa.

Spicer, L.J., 2004. Proteolytic Degradation of Insulin-Like Growth Factor Binding Proteins by Ovarian Follicles: A Control Mechanism for Selection of Dominant Follicles. Biology of Reproduction 70, 1223-1230.

Spicer, L.J., Santiago, C.A., Davidson, T.R., Bridges, T.S., Chamberlain, C.S., 2005. Follicular fluid concentrations of free insulin-like growth factor (IGF)-I during follicular development in mares. Domestic Animal Endocrinology 29, 573-581.

Vanholder, T., Opsomer, G., Kruif, A., 2006. Aetiology and pathogenesis of cystic ovarian follicles in dairy cattle: a review. Reproduction Nutrition Development 46, 105-119.

Table 1: Characteristics of mare follicular fluid (mean ± s.e.m) collected from normal preovulatory follicles (n=5), and cystic follicles (n=6).

Classification	Oestradiol pg/ml	Progesterone ng/ml	Testosterone ng/ml	IGF-I ng/ml	IGF binding
normal	65374 ± 27051.3	115.6 ± 93.1	4.02 ± 1.33	166.32 ± 21.7	3.61 ± 0.74
cystic	927 ± 526.7	5.4 ± 3.12	47.0 ± 16.86	107.64 ± 4.35	8.10± 1.20