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Animal Reproduction Science 63 (2000) 177–185

ANIMAL REPRODUCTION SCIENCE

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Amounts of selected coagulation factors in pre- and post-mortem follicular fluid are similar and do not correlate with molecular mass

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Received 15 July 1999; received in revised form 24 February 2000; accepted 5 May 2000

Abstract

This study was designed to evaluate the amounts of coagulation factors and to determine whether the protein profile in pre-ovulatory ovarian follicular fluid aspirated from ovaries collected from mares at slaughter are representative of that in follicular fluid collected from live animals. The proteins evaluated included, (i) albumin, ceruloplasmin and fibronectin, (ii) the procoagulant plasma proteins, Factor V (FV), Factor VII (FVII), Factor X (FX) and prothrombin, and (iii) the anticoagulant plasma proteins, antithrombin and α2-macroglobulin. The amounts of the individual proteins were similar in both types of follicular fluid. There was no correlation between the activity of FV, FVII, FX or prothrombin in follicular fluid and their molecular size although a correlation was found for the other proteins. These results suggest that the procoagulant proteins in follicular fluid are not likely derived from plasma. The total protein content of follicular fluid samples collected from both sources was similar and the results determined with the Biuret, Lowry and Biorad methods were also not significantly different ($P>0.05$).

Keywords: Horse; Follicular fluid; Plasma proteins; Procoagulant proteins

1. Introduction

Follicular fluid plays an important role in the physiology of follicular growth, oocyte maturation and ovulation (Gosden et al., 1988; Fortune, 1994). Although the factors that

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0378-4320/00/$ – see front matter
PII: S0378-4320(00)00178-0

regulate follicular development have not been fully elucidated, a group of proteins that may have a role in folliculogenesis are proteases, like thrombin, that have been shown to have mitogenic potential in cultured ovarian granulosa and theca cells (Forbes, 1996). In previous studies analyzing follicular fluid collected from ovaries obtained from an abbatoir, two of the proteins involved in thrombin generation, namely Factor VII (FVII) and Factor X (FX), were identified (Yamada and Gentry, 1995a). In plasma, FVII and FX circulate as inactive proenzymes which are converted to active serine proteases when thrombin generation is initiated (Davie et al., 1991; Gentry and Downie, 1993). The activated Factor X (FXa) combines with a protein cofactor, Factor V (FV), to convert inactive prothrombin to thrombin. Because previous studies had not evaluated follicular fluid for the presence of these two essential components of thrombin generation, one of the objectives of this study was to determine the amounts of FV and prothrombin in equine follicular fluid and to compare their activity levels with other procoagulant and anticoagulant proteins present in equine follicular fluid (Yamada and Gentry, 1995a; Gentry et al., 1996).

Previous studies reporting hemostatic proteins in follicular fluid have utilized follicular fluid collected from cows and mares at slaughter (Yamada and Gentry, 1995a,b). Because it is known that during apoptosis there are changes in cell membrane permeability which result in release of cellular contents (Brown et al., 1994), it was considered possible that post-mortem induced cellular degradation or alterations in vascular permeability might be the source of procoagulant proteins, and hence the thrombin generating and anticoagulant systems found in follicular fluid collected from the ovaries at slaughter might not be representative of the profile of fluid from live animals. Hence, a second objective of this study was to compare the activity of FV, FVII, FX and prothrombin in fluid (TFF) collected by transvaginal aspiration of mares with fluid (AFF) collected from ovaries obtained from an abattoir. The comparison of the protein profile in the two types of fluids was extended to include the anticoagulant proteins, antithrombin and α2-macroglobulin (α2-M) and other plasma proteins, including albumin, ceruloplasmin and fibronectin, which have previously been evaluated only in fluid from pre-ovulatory follicles isolated from post-mortem ovaries (Yamada and Gentry, 1995a; Gentry et al., 1996).

A third objective of this study was to compare various methods of determining the total protein content of follicular fluid because it has been suggested that biochemical constituents present in human follicular fluid can interfere with several of the commonly used assay methods (Gonzales et al., 1992). For example, the total protein content of a follicular fluid sample may be overestimated with the Biuret reagent if small peptides are present, and in the Lowry (Folin phenol) assay if free phenol groups, as in estradiol molecules, are present (Gonzales et al., 1992). For this study, the Biuret, Lowry and Biorad methods were each used to determine the total protein content of follicular fluid collected from live and slaughtered mares.

2. Materials and methods

2.1. Collection and preparation of follicular fluid

Follicular fluid from mares was collected by follicle aspiration using a transvaginal ultrasound-guided technique (Bruck et al., 1992). Prior to aspiration, the follicular diameter was determined by ultrasonography. A total of 10 follicles were aspirated from six

mares. Ovaries were collected at slaughter from mares of unknown reproductive histories, the diameter of the follicles determined by caliper measures and follicular fluid aspirated as previously described (Gentry et al., 1996). Follicular fluid collected from individual follicles by either technique was centrifuged in a plastic tube at $3000 \times g$ for 15 min at 4°C and the clear supernatant transferred, in several aliquots, to clean vials and frozen at −20°C. Ten samples from each group were assayed in duplicate, and the results for the analysis of total protein and individual proteins calculated as mean±S.E.

2.2. Preparation of plasma

Blood was collected from 10 healthy mares, by venepuncture of the external jugular vein, into a plastic syringe containing 0.17 M sodium citrate in the proportion nine parts blood to one part anticoagulant. The mares were housed at the University of Guelph research farm according the guidelines of the Canadian Council on Animal Care. The citrated blood was transferred to plastic centrifuge tubes, centrifuged at $3000 \times g$ for 20 min at 4°C and the top two-thirds of the resulting platelet poor plasma (PPP) removed. The PPP from each animal was pooled and the resulting sample stored in aliquots at −20°C.

2.3. Protein determinations

The total protein content of each of the follicular fluid samples was assayed using the Biuret assay (Peters, 1968), the Lowry assay (Lowry et al., 1951) and the Biorad assay (Bio-Rad Laboratories, Richmond, CA) while albumin was determined using the Bromcrescol Green assay as previously described (Gentry et al., 1996). Rocket immunoelectrophoresis was used to assess fibronectin, α2-macroglobulin and ceruloplasmin levels as previously described using pooled equine plasma as the standard (Gentry et al., 1996).

The specific coagulation factor assays for FV, FVII and FX were performed with a semi-automated system (Coag-A-Mate XM, Organon Teknika, Durham, NC) as previously described (Yamada and Gentry, 1995a). Briefly, the procedure involves mixing a diluted sample of follicular fluid with plasma deficient in the protein being assessed (BioPool Canada Inc., Burlington, Ont.), activating the system with a mixture of recombinant human tissue factor, phospholipid and calcium (Dade-Behring Inc., Miami, FL) and measuring the effectiveness of the follicular fluid sample to correct the clotting time compared with the normal equine reference plasma. A chromogenic assay was used to determine prothrombin activity using *E. carnis* venom (Ecarin, Sigma Chemical Co., St. Louis, MO) to directly convert prothrombin to thrombin (Franza et al., 1975). The esterolytic activity of the thrombin generated was determined with a chromogenic substrate, S-2238 (Helena Laboratories, Beaumont, TX) and the absorbance recorded at 405 nm converted to percentage activity from a standard curve prepared with the equine reference plasma. A chromogenic assay using the S-2238 substrate was also used, as previously described (Gentry et al., 1992), to determine antithrombin activity compared to equine reference plasma.

2.4. Statistical analysis

Statistical analyses were conducted out using a SigmStat computer program (Jandel Scientific, San Rafael, CA).

3. Results

3.1. Selection of follicles

Only fluid (AFF) from abattoir source follicles between 30 and 59 mm in diameter was used in this study to allow comparison with previous studies on the protein content and hormonal profile of fluid of follicles of this size (Pierson and Ginther, 1985; Meinecke et al., 1987; Yamada and Gentry, 1995a; Gentry et al., 1996). The follicular fluid collected transvaginally (TFF) was from follicles estimated to be pre-ovulatory and varying between 20 and 47 mm in diameter.

3.2. Comparison of total protein content of follicular fluid

The average total protein values were similar for the TFF and AFF samples irrespective of whether the total protein was determined by the Biuret, Lowry or Biorad assays (Table 1). Using the combined data from all three assays, the mean total protein in the TFF samples was 55.0 g l^{-1} while that of the AFF samples was 58.5 g l^{-1}. Although the range of values for total protein was larger with each assay procedure for the AFF samples compared with the TFF samples (40.8–74.2 g l^{-1} compared with 50.1–64.7 g l^{-1}), overall there was no statistically significant difference among any of the results ($p>0.05$).

3.3. Comparison of protein profiles of transvaginal and abattoir follicular fluid samples

The activity of FV, FVII, FX and prothrombin were used to evaluate the thrombin generating potential in all follicular fluid samples. As shown in Table 2, there was no statistically significant difference ($p>0.05$) between the amount of each of these proteins in the TFF and AFF samples although there was a wide range in the activity of the various proteins. For example, in both TFF and AFF, prothrombin was present at approximately 70% of equine plasma concentrations while the protein cofactor, FV, was present at between 1–2%. Likewise, there was no difference in the overall thrombin modulating activity, as estimated by the amount of antithrombin, α2-macroglobulin and ceruloplasmin, between TFF and AFF (Table 2). Relatively greater amounts of both antithrombin and ceruloplasmin, between 80 and 85% of plasma concentrations, were found in both types of follicular fluid while α2-macroglobulin was present at much lower concentrations, around 9–11%

Table 1
Comparison of the total protein concentration in follicular fluid samples collected transvaginally ($n=10$) or by aspiration of ovaries collected post-mortem (abattoir samples, $n=10$) as determined by three different assay methods[a]

Protein (g l^{-1})	Transvaginal samples (range)	Abattoir samples (range)
Total protein (Biuret method)	56.2±2.6 (50.4–64.7)	60.6±6.3 (43.0–68.2)
Total protein (Lowry method)	54.0±3.8 (50.3–61.0)	60.5±8.1 (46.6–74.2)
Total protein (Biorad method)	54.7±3.8 (50.1–61.3)	54.3±4.8 (40.8–65.4)

[a] Values are reported as mean±S.E.

Table 2
Individual protein concentration in follicular fluid samples collected transvaginally (n=10) or by aspiration of ovaries collected post-mortem (abattoir samples, n=10) expressed as a percentage of the protein concentration in normal equine plasma[a]

Protein	Transvaginal samples (% of normal plasma)	Abattoir samples (% of normal plasma)	Molecular weight of protein (kDa)
Thrombin generating system			
Factor V	0.9±0.2	1.9±0.6	330
Factor VII	25.3±2.7	22.3±3.0	50
Factor X	25.3±4.3	23.7±4.5	55
Prothrombin	69.2±4.0	69.7±6.4	70
Thrombin modulating system			
Antithrombin	82.9±3.7	82.1±5.9	52
α2-macroglobulin	11.0±4.2	9.4±1.8	725
Ceruloplasmin	84.6±5.2	79.6±2.3	132

[a] Results are expressed as mean±S.E.

of plasma. The amount of albumin in the TFF and AFF samples was similar at 33.8±0.7 and 33.2±1.1 g l^{-1}, respectively, as was the amount of fibronectin which was found to be 34.2±4.0 and 38.0±8.0% of that of plasma, respectively.

3.4. Comparison of filtration fraction of individual proteins and size in follicular fluid

The relative concentration of individual proteins in follicular fluid relative to the percentage of the equivalent protein in homologous plasma has been used to determine theoretical filtration percentages or the filtration fraction of individual proteins (Gonzales et al., 1992). Using this relationship to compare the filtration fraction of the group of proteins evaluated in TFF and AFF, it is apparent that there is no direct correlation between molecular size (kDa) and activity for the group of proteins involved in thrombin generation, namely, FV, FVII, FX and prothrombin (Fig. 1). However, for other proteins determined in this study, including the thrombin regulatory proteins, antithrombin and α2-M, there did appear to be some correlation between concentration and molecular size (Fig. 1).

4. Discussion

This study has demonstrated that the protein profile is similar in follicular fluid collected transvaginally from live mares and in fluid aspirated from follicles of abattoir source ovaries. The relative amounts of total protein, albumin, antithrombin, ceruloplasmin, fibronectin and α2-M found in the abattoir samples in this study were similar to those previously reported for follicular fluid derived from pre-ovulatory follicles from slaughtered mares (Gentry et al., 1996). Although hormonal analysis of follicular fluid samples was not performed in this study, we have previously shown that there is no correlation between the concentration of albumin, antithrombin, ceruloplasmin, fibronectin and α2-M and estradiol-17β, progesterone or androstendione concentration in follicular fluid samples (Gentry et al.,

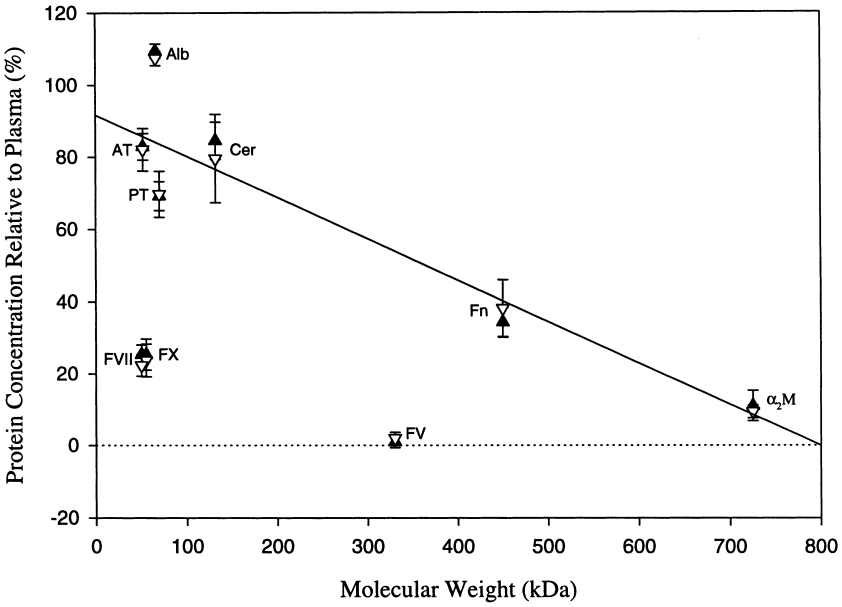

Fig. 1. Relationship of the molecular weight and the concentration (mean±S.E.) of proteins in follicular fluid collected transvaginally from mares (closed triangles) or from ovaries collected at an abattoir (open triangles). Only when values for AT, Alb, Cer, Fn and α2-M were used could a regression line be determined. AT: antithrombin; Alb: albumin; Cer: ceruloplasmin; FV: Factor V; FVII: Factor VII; FX: Factor X; Fn: fibronectin; Pt: prothrombin; α2-M: α2-macroglobulin.

1996). Likewise, there was no difference in the activity of proteins involved in thrombin generation, namely FV, FVII FX and prothrombin between the two types of fluid (Table 2).

The results of this study demonstrate that not only is prothrombin present in follicular fluid but that the same initial activation pathway that is essential for thrombin formation in plasma, namely FV, FVII and FX, is also present. Since FV, FVII, FX and prothrombin have also been found in physiologically relevant amounts in human, bovine, ovine and porcine follicular fluid, it would appear that this thrombin generating pathway is common to all mammalian follicular fluid (Gentry et al., 2000; Semotok, 1999). The physiological role(s) for thrombin within the follicle have not yet been fully elucidated, but we have hypothesized that thrombin regulates folliculogenesis through its 'growth factor-like' activity that is mediated through specific thrombin receptors belonging to the seven transmembrane G-protein coupled receptor family (Hung et al., 1992; Coughlin, 1993; Grand et al., 1996). For example, not only is thrombin receptor mRNA present in bovine granulosa and theca cells but thrombin elicits a strong mitogenic response in cultured theca cells with a potency similar to that of epidermal growth factor and fibroblast growth factor (Forbes, 1996). However, this may not be the only role for thrombin within the follicle since, in various cell culture systems, it has been shown to promote a number of cellular responses. In addition to cell proliferation and migration, these functions include the induction of protein synthesis and secretion and the regulation of membrane permeability (Malik and Fenton, 1992; Chen

et al., 1994; Grand et al., 1996; Goldsack et al., 1998). In contrast to plasma where the primary role of thrombin is the conversion of soluble fibrinogen to the insoluble fibrin that forms the matrix of a blood clot (Gentry and Downie, 1993), it is unlikely that the function of the thrombin generating system within a healthy follicle is to induce the formation of a fibrin gel. Although equine follicular fluid contains fibrinogen at approximately 40% of the amount found in plasma (Yamada and Gentry, 1995a), fibrin strand formation is not observed in fluid from healthy follicles even when the fluid is exposed to conditions such as allowing the follicular fluid to interact with a glass surface at 22°C for 18 h, that result in fibrin formation in plasma (unpublished observations). The absence of fibrin formation in follicular fluid may be due, at least in part, to the relative activity of the thrombin generating system and the thrombin modulatory system (Table 2). It is apparent that in follicular fluid, the balance of activity is directed towards inhibition of thrombin activity, whereas, in plasma, conditions are designed to rapidly generate high local concentrations of thrombin at sites of vascular damage. In this study, as in a previous report (Yamada and Gentry, 1995a), the level of the antiproteinase, antithrombin, in follicular fluid is closer to that of plasma than any of the proteinases, such as FX. The relatively greater amount of antithrombin in equine follicular fluid may explain the results of an earlier study that demonstrated the ability of equine follicular fluid to inhibit the clotting of plasma in a dose dependent manner (Stangroom and Weevers, 1962) since antithrombin is capable of inhibiting both the formation of thrombin and the thrombin proteolytic activity (Gentry and Downie, 1993). It is also possible that the relatively greater amounts of ceruloplasmin present in follicular fluid may contribute to this anticoagulant property since it is known that, in addition to its role as an antioxidant, ceruloplasmin can regulate thrombin generation through enhancement of the FV-protein C anticoagulant system (Walker and Fay, 1990).

It has been shown for a number of mammalian species that, for plasma proteins present in follicular fluid, there is a correlation between molecular size of the protein and its concentration in follicular fluid (Andersen et al., 1976; Wise, 1987; Nagy et al., 1989; Suchanek et al., 1990; Gonzales et al., 1992; Gentry et al., 1996; Collins et al., 1997). This study has also demonstrated a similar correlation for proteins, such as albumin, ceruloplasmin, fibronectin and α2-M (Fig. 1). However, no such correlation exits for the amounts of the protein components of the thrombin generating system (Table 2, Fig. 1). In contrast, in synovial and lymph fluids, procoagulant proteins demonstrate an inverse relationship between their concentration and molecular weight (Chang et al., 1995; Le et al., 1998). The implication of these results is that the thrombin generating system present in follicular fluid is not derived from the filtration of plasma proteins into the antrum but rather from local synthesis and secretion. This hypothesis is supported by preliminary evidence that bovine granulosa cell lysates possess both prothrombin mRNA and protein, indicating the potential for procoagulant protein synthesis in these cells (Schroeder et al., 1999).

In addition to the similarity of the procoagulant system in follicular fluid collected from live animals or at post-mortem, the total protein content of the samples is similar and the values obtained are independent of the methodology (Table 1). The ratio of total protein in FF/serum was on average 0.78 for the transvaginally collected follicular fluid, which was similar to the values of 0.74 for both pony and human follicular fluid similarly collected (Gonzales et al., 1992; Collins et al., 1997). The average ratio of total protein in FF/serum was slightly higher, at 0.83, in the abattoir samples but, because of the wider range of values

found for this group, the average results were not significantly different ($p>0.05$) from that of the samples collected from the live mares. The results of the present study indicate that follicular fluid samples collected from pre-ovulatory follicles at post-mortem are suitable for investigating the protein composition and potential role of proteinases and proteinase inhibitors in follicular fluid.

Acknowledgements

The assistance of Dr. Claire Plante and Dr. Lee Morris, Department of Population Medicine, University of Guelph, in the transvaginal aspiration of equine follicular fluid is gratefully acknowledged as is the expert technical assistance of Michelle Ross. This research was supported by the Natural Sciences and Engineering Research Council of Canada and the Ontario Ministry of Agriculture, Food and Rural Affairs.

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