PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 167, 290–292 (1981)

Effects of Lyophilization and Freezing on the Analysis of Mouse Uterine Lumenal Fluids and Qualitative Aspects of Secretions from Breeder and Nonbreeder Mice (41166)

M. B. GUISE, F. C. GWAZDAUSKAS, AND J. A. LINEWEAVER

Department of Dairy Science, College of Agriculture and Life Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Abstract. Uterine flushings were obtained from 160 ICR albino breeder and nonbreeder mice classified as either in estrus or in the nonestrus stage of cycle to evaluate proteins by electrophoresis. In the initial experiment, uterine flushings were frozen or lyophilized before disc–gel electrophoresis. Lyophilization resulted in a significant loss of protein constituent from the samples. In a second experiment using only frozen samples, fewer protein bands were found following disc–gel electrophoresis in estrous flushing from mice that had previously littered than in estrous flushing of nonbreeder mice which had been previously exposed to males and had not conceived. There appears to be qualitative differences in γ protein peaks, with nonbreeder mice having fewer peaks during estrus and an excess of an α peak protein during the nonestrual phase of the cycle. The excess α protein during nonestrus may be indicative of a hostile uterine environment and be related to lowered reproductive efficiency.

Uterine secretions play an important role in the development of the early embryo. *In vitro* experimentation demonstrated both beneficial and detrimental effects of several protein components on morula to blastocyst development (1). Additionally, Hamana and Hafez (2) suggested that uteroglobin played a role in embryonic development of rabbit embryos between the shedding of the zona pellucida and implantation. Blastocyst development, including radial expansion and dissolution of the mucin coat, required the action of uterine secretory proteins (3). Several methods have been proposed for handling proteins prior to quantitative and qualitative assessments (4). Methods of handling uterine fluids before electrophoretic separation of protein components require further evaluation because of conflicting results on proper sample preparation technique (5, 6). The present study was designed to evaluate variation of profiles between murine uterine flushings which had been either frozen and lyophilized or just frozen and to assess uterine protein profiles in breeder and nonbreeder mice.

Materials and Methods. A total of 160 female random bred ICR albino mice were assigned to an unbalanced nested factorial design. Mice were housed under a controlled 14:10 (light:dark) hour light cycle and maintained at 25°. Females were classified as breeders or nonbreeders on the basis of their reproductive status following exposure to fertile males for a 15-day breeding period. Breeder mice were those that had previously littered and were obtained after weaning, whereas, nonbreeders were females which failed to conceive during the breeding period.

Females were classified as either estrus or nonestrus by visual examination of the external genitalia at the time of sacrifice. Estrual mice exhibited bright red swollen vulvas, while nonestrual mice had pale and nonedematous vulvas. Stages of the cycle were further verified by noting uterine vascularization at the time of uterine horn excision.

Following classification, mice were anesthetized with methoxyflurane[1] and decapitated. Animals were positioned in dorsal recumbancy, the uteri were excised, and attached adipose tissue and cervix were removed. Each uterine horn was flushed retrograde with 0.2 ml 0.9% sodium

[1] Pitman–Moore Company.

0037-9727/81/060290-03$01.00/0

chloride solution using a 23-gauge needle attached to a 1-ml tuberculin syringe; flushings were collected in a 2-ml disposable beaker, and frozen immediately at −68°. Frozen samples to be lyophilized were placed in a glass dish and covered with two layers of cheesecloth. The dish was placed on a −40° prefrozen shelf of a freeze dryer (Virtis 10-145 MR-BA). An internal vacuum atmosphere of 100 μm Hg was created and the samples dried at 60° for 12 hr with the condenser continually operating at −40°. Lyophilized samples were stored at 4° until electrophoresis.

Uterine flushings were subjected to polyacrylamide discontinuous gel (5-mm columns) electrophoresis (7) in a Hoefer electrophoretic apparatus. A two-gel system was employed. The 7.5% separating gel was composed of acrylamide, buffer (1 *N* HCL, Tris, *N,N,N*^{1},*N*^{1}-tetramethylethylenediamine, water), deionized water, and ammonium persulfate in a ratio of 2:1:1:4. Gels were cast by pipetting 1 ml of separating gel solution into each gel tube and placed into 4° for 40 min for chemical polymerization. The 3.5% stacking gel was made of acrylamide, buffer, riboflavin, and sucrose in a ratio of 2:1:1:4. Stacking gels were cast by pipetting 0.5 ml of the gel solution over the polymerized separating gel. The assembly was placed in a fluorescent light source at 4° for 40 min for photopolymerization. Lyophilized samples were massed and reconstituted to 0.2 ml with deionized water. Fifty and 100-μl aliquots of the lyophilized (n = 97) and frozen (n = 63) uterine flushings were placed on the gel. Following a 4-hr migration, (36 amp), gels were stained with 0.08% Coomassie brilliant blue for 1 hr at 37°. After destaining with 7.5% acetic acid and 5.0% methanol in water, the 100-μl sample gels were scanned at 600 nm with a Gelman DCD-16 computing densitometer and protein bands were determined. In preliminary trials, peak numbers of different standard concentrations of protein did not differ because of staining intensity. Protein bands were quantified based on visual determination of protein peaks from the densitometric tracings. Qualitative estimates of mouse uterine proteins were determined based on cellulose acetate electrophoretic bovine serum densitometer profiles.

Statistical analysis. Statistical analyses were by the Statistical Analysis System procedures (8). Least-squares analysis of variance was the major method employed. The t statistic was used to evaluate specific mean differences (9).

Results and Discussion. *Freezing vs lyophilization.* Qualitative protein profiles generated by densitometric analysis of disc–gels were compared between lyophilized and frozen samples (Table I). Analysis of variance revealed a significant sample handling effect ($P < 0.01$) and interaction ($P < 0.05$) of breeding status and method of sample handling. Lyophilized samples from breeders had 1.2 more protein bands than those from nonbreeders; however, frozen samples from nonbreeders had two bands not found in frozen breeder samples. Significantly lower numbers of protein bands from lyophilized uterine secretions suggested a loss of protein quality available for electrophoretic separation. These data agree with those of Roberts and Parker (6), who reported that lyophilization of bovine uterine proteins resulted in a loss of uterine protein by irreversible adsorption onto glass containers. Additionally, Fridlansky and Milgrom (10) and Arthur *et al.* (11) found reduced progesterone binding to rabbit uteroglobin following lyophilization and dialysis, whereas freezing had no effect. These observations suggest that the procedures had altered the structure of the protein or caused a reduction in available protein. Based on these findings, subsequent evaluations of disc–gel electrophoretic analyses were performed utilizing frozen samples.

TABLE I. NUMBER OF PROTEIN BANDS ASSOCIATED WITH BREEDING STATUS AS RELATED TO METHOD OF HANDLING OF MOUSE UTERINE FLUSHINGS

Breeding status	Lyophilization[a]	Frozen
Breeder females	7.1 ± .5 (44)[b,c]	11.3 ± 0.6 (37)[e]
Nonbreeder females	5.9 ± .4 (20)[d]	13.3 ± 0.5 (26)[f]

[a] Only 64 of 97 samples had adequate protein for qualitative determinations.

[b] Mean ± SE; number of flushings in parentheses.

[c–f] Means with different superscripts in the same row and column are different ($P < 0.05$).

TABLE II. DISC-GEL ELECTROPHORETIC PROTEIN BAND NUMBERS AND QUALITATIVE ASPECTS OF PROTEIN PEAKS ASSOCIATED WITH BREEDING CLASSIFICATION AND STAGE OF ESTROUS IN FROZEN MOUSE UTERINE FLUSHING SAMPLES

Breeding Status	Estrous stage	Protein bands[a]	Protein peak[c]			
			Albumin	α	β	γ
Breeder females	Estrus	11.9 ± 0.7 (16)[b,c]	100	56	81	50
	Nonestrus	10.8 ± 0.9 (21)[b]	100	29	67	52
Nonbreeder females	Estrus	14.0 ± 0.9 (7)[d]	100	43	86	14
	Nonestrus	13.0 ± 0.7 (19)[c,d]	100	63	84	37

[a] Mean ± SE; number of scanned gels in parentheses.
[b-d] Means with same superscript are not different ($P < 0.05$).
[c] Percentage of gels with the particular protein peak.

Uterine secretory proteins from breeder and nonbreeder mice. The numbers of protein bands following disc-gel densitometric analysis from breeder and nonbreeding uterine flushings from the different estrous cycle classifications are in Table II. Analyses of variance revealed significant ($P < 0.05$) differences in the number of protein bands between the breeder and nonbreeder females. Densitometric qualitative aspects of disc-gels showed subtle changes in α, β, and γ band appearance between breeder and nonbreeder flushings (Table II). Qualitative differences in uterine secretory protein profiles between breeder and nonbreeder female mice suggest alterations in the γ peak in the samples collected at estrus. Additionally, the occurrence of the α region of nonestrus nonbreeder flushings in approximately twice the proportion of the nonestrus breeder samples suggests a uterine environment incompatible with embryo development near the time of implantation when uterine luminal proteins may be activating the embryo (12). If the α region was involved with overproduction of uteroglobin, subsequent embryo development could be affected (13). Alterations in the protein bands appearing in the disc-gels may be related to endocrine events. Fertilization of ova in nonbreeder female mice crossed with males of breeder lines was not different from female mice lines which have no reproductive inefficiency (14).

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Received July 14, 1980. P.S.E.B.M. 1981, Vol. 167.