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A Letter From Dr. Hugh Hildebrandt

In this issue of the fur animal research newsletter I will try to incorporate a few changes that some of you have requested. I will try to highlight the primary terminology when used for the first time in the article in order to be found easier when abbreviations are used later in the article. I would also like to point out that some research has a very extensive list of references attached. The list shows just how broad of an information base that researchers draw from in doing studies for our industry. Browsing these lists may peak your interest in other articles already published and are usually available somewhere online.

I would like to thank Dr. Gorham for his article on influenza for this issue and would like to say it was very timely as we now have an influenza case in the US documented in this issue.

On the research front this summer we have a number of great projects one of which is a continuation of some AD vaccination work findings that were never followed up from years ago. This info had been brought up at a number of meetings and we finally have the research in the works this summer. I hope to report on that in the next issue. Another development you will start hearing about is the Q ELISA test. This development stems from the R-1 and R-2 and Progressive designation for mink with AD I discussed in the first issue. The Q ELISA has a new and innovative blood collection method and should help the industry with AD testing and also truly speed up the selection for R-1 mink. I hope to have some research results for the next issue.

Dr. Hugh Hildebrandt

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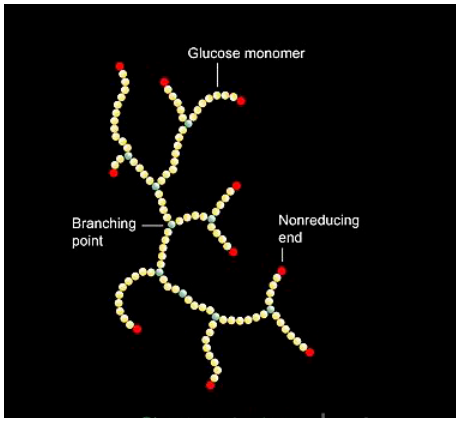

The effects of estradiol and catecholestrogens on uterine glycogen metabolism in mink (Neovison vison)

Abstract

Glycogen is a uterine histotroph nutrient synthesized by endometrial glands in response to estradiol. The effects of estradiol may be mediated, in part, through the catecholestrogens, 2-hydroxycatecholestradiol (2-OHE2) and 4-hydroxycatecholestradiol (4-OHE2), produced by hydroxylation of estradiol within the endometrium. Using ovariectomized mink, our objectives were to determine the effects of estradiol, 4-OHE2, and 2-OHE2 on uterine: 1) glycogen concentrations and tissue localization; 2) gene expression levels for glycogen synthase, glycogen phosphorylase, and glycogen synthase kinase-3B; and 3) protein expression levels for glycogen synthase kinase-3B (total) and phospho-glycogen synthase kinase-3B (inactive). Whole uterine glycogen concentrations (mean ± SEM, mg/g dry wt) were increased by estradiol (43.79 ± 5.35), 4-OHE2 (48.64 ± 4.02), and 2-OHE2 (41.36 ± 3.23) compared to controls (4.58 ± 1.16; $P \leq 0.05$). Percent glycogen content of the glandular epithelia was three-fold greater than the luminal epithelia in response to estradiol and 4-OHE2 ($P \leq 0.05$). Expression of glycogen synthase mRNA, the rate limiting enzyme in glycogen synthesis, was increased by 4-OHE2 and 2-OHE2 ($P \leq 0.05$), but interestingly, was unaffected by estradiol. Expression of glycogen phosphorylase and glycogen synthase kinase-3B mRNAs were reduced by estradiol, 2-OHE2, and 4-OHE2 ($P \leq 0.05$). Uterine phospho-glycogen synthase kinase-3B protein was barely detectable in control mink, whereas all three steroids increased phosphorylation and inactivation of the enzyme ($P \leq 0.05$). We concluded that the effects of estradiol on uterine glycogen metabolism were mediated in part through catecholestrogens; perhaps the combined actions of these hormones are required for optimal uterine glycogen synthesis in mink.

Keywords: Uterus; Glycogen; Glycogen synthase (Gys); Glycogen synthase kinase-3B (Gsk3B), Glycogen phosphorylase (Pyg); Catecholestrogen; Estradiol

1. Introduction

Mink exhibit obligatory embryonic diapause and may have blastocysts up to 60 d of age (*post coitum*) at implantation, giving birth to as many as 17 offspring [1,2]. Until formation of the placenta is complete, embryonic growth and development depend on uterine glandular secretions or histotroph, containing enzymes, hormones, growth factors, and nutrients [3,4].

Uterine histotroph is rich in a variety of carbohydrates, including glycogen [5–7]. In anestrous mink, uterine glycogen deposits were detected in luminal but not glandular epithelia [8]. During estrus and embryonic diapause, glycogen deposits were detected in luminal and glandular epithelium, and decreased after implantation [9–11]. The post-implantation reduction in uterine glycogen content, which

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has also been reported in cats, [12] armadillos [13] and ferrets [14], is thought to reflect usage of the nutrient by embryos.

Glycogen synthesis is catalyzed by glycogen synthase, whereas catabolism is controlled by glycogen phosphorylase [15]. The enzyme glycogen synthase kinase-3B, which is constitutively active in uterine tissue [16], phosphorylates glycogen synthase, reducing its activity and glycogen synthesis [17]. Similarly, phosphorylation and inactivation of glycogen synthase kinase-3B reduces inhibition on glycogen synthase, leading to increased glycogen synthesis.

Glycogen synthesis in the uteri of rats, rabbits, and guinea pigs is increased by estradiol [18–21]. Moreover, the effects of estradiol may be mediated in part, through the catecholestrogens, 2-hydroxycatecholestradiol (2-OHE2) and 4-hydroxycatecholestradiol (4-OHE2), following hydroxylation of the parent hormone by the uterine endometrium [22–24]. In the mouse, 4-OHE2 activated dormant blastocysts [25] and upregulated expression of the lactoferrin gene in the uterus [26]. Catecholestrogens bound to conventional receptors for estradiol, but may also act through distinctly separate signaling pathways [23,26]. Due to their rapid metabolic clearance, it is unlikely that catecholestrogens function as circulating hormones, but rather act as autocrine, paracrine, and intracrine mediators of the effects of estradiol [23,27–29]. In the present study, we tested the hypothesis that the effects of estradiol on uterine glycogen metabolism in the mink may be mediated in part through catecholestrogens.

2. Materials and methods

2.1. Animals and treatments

Twenty-four adult (15 to 16 mo old) female mink were moved to the indoor animal facility on the Idaho State University (ISU) campus during late August. All mink were primiparous, high producers, having given birth to a litter of 6 to 8 offspring the previous spring. Animals were housed individually, fed a mixture of chicken and fish by-products daily, received water *ad libitum,* and exposed to a photoperiod approximating natural changes in day length for Southeastern Idaho, with an Electronic Astronomic Time Switch, Model ET816CR (Intermatic Corp., Spring Grove, IL, USA). Mink were illuminated with General Electric Full Spectrum Chroma-50, Model F40C50 lamps, and maintained at a room temperature of 25 ± 3 °C. Animal care and research procedures were approved by the Institutional Animal Care and Use Committee of ISU, and complied with the Guide for the Care and Use of Laboratory Animals.

Between September 6 and 7 (Day 0), all mink were bilaterally ovariectomized through a single mid-ventral incision while under ketamine hydrochloride anesthesia (50 mg/kg body weight; Fort Dodge Animal Health, Ft. Dodge, IA, USA), and returned to their cages to recover and allow natural elimination of residual ovarian hormones. Subsequently, mink (N = 6/group) were injected twice daily (0600 and 1400) on Days 12, 13, and 14 (Sept 18, 19, 20) with 200 μg/kg body weight of estradiol, 4-OHE2, or 2-OHE2 (R187933, H4637, H3131 respectively; Sigma Chemical Co., St. Louis MO, USA), in sesame seed oil, whereas control mink received oil injections only. On Day 15, each mink was anesthetized with ketamine hydrochloride, the uterus removed, weighed, and quick frozen in liquid nitrogen. All animals were then killed with a lethal dose of Sleep-A-Way (Fort Dodge Animal Health).

2.2. Glycogen determination for uterine homogenates

Uterine glycogen concentrations were determined according to Passonneau and Lauderdale [30]. A uterine sample (50 mg) from each animal was lyophilized, and homogenized in 0.03 M HCl (Sample A). To a 50 μL aliquot of Sample A, 200 μL of 1.0 M HCl was added, followed by incubation at 100 °C for 4 h (Sample B), to break glycogen down to glucose. Glucose was detected using infinity glucose hexokinase reagent (TR15498; Fisher Scientific, Pittsburgh, PA, USA) and quantified spectrophometrically (λ = 340 nm), by comparing unknowns against a standard curve of increasing glucose concentrations. Total glycogen concentrations were determined by subtracting the free glucose concentration of Sample A from the total glucose concentration of Sample B.

2.3. Glycogen detection in uterine cells and tissues

Uterine samples were fixed in 10% neutral buffered formalin, dehydrated and mounted in paraffin. Three independent cross sections (4 μm thick) from each uterus were stained with Period-Acid-Shiff (PAS) reagent to detect glycogen deposits, and counter-stained with hematoxylin. Duplicate sections serving as negative controls were pretreated with diastase (Sigma Chemical Co.) to digest glycogen to glucose, prior to PAS staining. Digital images were subsequently captured at 25 and 400×, and analyzed in triplicate for each animal using ImageJ software (1.43h, Wayne Rasband WS, US National Institute of Health, NIH, Bethesda, MD, http://rsb.info.nih.gov/ij/). To provide de-

Table 1
Primers used for qPCR of mink uterine gene transcripts and amplicon characteristics.

Genes	Forward & reverse primers (5′ to 3′)	Amplicon (bp)	Melt temperature (°C)	Accession No.*
Gsk3B	CTTGCGGGAGAACTAATGCTG CCAATGACTTTAGTGTCTGTGTAACTG	295	83.3 ± 0.03	NM_032080
Pyg	GTCAGAACAGATCTC CAC TGCTGG GTCTTTGAAGAGGTCTGGCTGATTGG	296	81.5 ± 0.09	NM_012638.1
Gys	AGTCCTCAGAGCAGC GATGTGG GTACCATCACAGTACGGTGACACATG	327	77.4 ± 0.23	NM_001109615.1
B-Actin	GATGACC CAGATCATGTTCGAG CCATCTCCTGCTCGAAGTCC	329	86.0 ± 0.03	NM_031144

Gys, glycogen synthase; Pyg, glycogen phosphorylase; Gsk3B, glycogen synthase kinase-3B; B-Actin, beta actin.
* http://www.ncbi.nlm.nih.gov.

tailed illustrations of positive PAS staining in various uterine cell types as well as endometrial gland lumens, a third set of images were captured at 1000×, under oil immersion.

Total endometrial and myometrial areas were measured by delineating each area at 25× with an Intuos electronic pen tablet (Wacom Corp., Vancouver, WA, USA) and expressing the area as total pixel count using the ImageJ autothreshold plugin. Detection of glycogen deposits, based on positive PAS staining (magenta color), was accomplished with the ImageJ colour threshold plugin (v. 1.11). The color intensity standard for glycogen deposits was established using PAS-stained rat liver sections. An average of 10 rat liver color intensity measurements for positive PAS staining were obtained and the mean value of these measurements was used as the standard color threshold against which all uterine sections were analyzed. All PAS staining intensity values after diastase treatment were subtracted from those without diastase, when calculating glycogen content.

The total glycogen content of the endometrium and myometrium were determined for complete uterine cross sections at 25×, using ImageJ and expressed as total PAS positive pixel counts. To determine glycogen content of glandular and luminal epithelia, required viewing images at 400×. Because of steroid-induced enlargement of the uterus, the number of glands that could be detected at 400×, varied from as few as six per section in estradiol-treated mink to as many as 20/section in control mink. We therefore normalized glycogen content values for the glandular and luminal epithelia by dividing the total number of positive PAS pixel counts for glycogen by the total pixel count for the area sampled and expressed the data as percent glycogen content. Data are also presented as relative fold-increase compared to controls.

2.4. Total RNA isolation

Total RNA was isolated from 25 to 50 mg uterine tissue from each mink using the QIAGEN RNeasy® Fibrous Tissue Mini Kit (74704, QIAGEN, Valencia, CA, USA). Samples were screened for protein contamination by measuring light absorption of each sample at 260 nm (DNA and RNA) and 280 nm (protein). Only RNA preparations with 260/280 ratios of 1.9 or greater were used for qPCR analysis.

2.5. Primer design for quantitative polymerase chain reaction (qPCR)

Since nucleotide sequences for mink target genes are unknown, we compared each target gene sequence in the rat with those of other species using the Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI; NIH http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome). Regions of the rat genome for glycogen synthase (Gys), glycogen phosphorylase (Pyg) and glycogen synthase kinase-3B (Gsk3B) that were 95–100% homologous for many species (i.e., mouse, dog, and human), were used to design primers for qPCR (Table 1), which were purchased from Integrated DNA Technologies (Coralville, IA, USA).

2.6. Production of first strand transcripts

Conversion of unstable RNA to stable cDNA transcripts (first strand transcripts) was achieved using a reverse transcriptase generated from the Moloney Murine Leukemia Virus (Promega, Madison, WI, USA), and random hexamer primers, according to the manufacturer's instructions.

2.7. Quantitative PCR (qPCR)

Reactions were carried out in triplicate using Fast Start SYBR Green Master Mix (04-673-514-001;

Roche Applied Science, Indianapolis, IN,USA), containing forward and reverse primers at 4 μM each + cDNA template at 100 to 200 ng. Each sample was subjected to 40 alternating cycles of a three-segment amplification program: 1) 15 s denaturation at 95 °C; 2) annealing for 1 min at 55 °C (Gsk3B and B-Actin) or 60 °C (Gys and Pyg); and 3) elongation at 72 °C for 1 min. The PCR products (amplicons) were detected in real time, by measuring SYBR-green fluorescence during the annealing stage, with the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Efficiencies of amplicon doubling during each PCR cycle were: B-Actin: 100%, Gsk3B: 82%, Gys: 99% and Pyg: 83%. Negative controls consisted of samples with no template and amplification was never above background, indicating a lack of non-specific amplification. Primer specificity was validated by melt-curve analysis for each amplicon, which yielded a single melting temperature for each gene product (Table 1).

Data were analyzed using the Relative Standard Curve Method for Quantification of gene products (Applied Biosystems Chemistry Guide for Real Time PCR, NO. 4348358). Standard curves specific for each amplicon, were generated from three pooled control mink uteri. The cDNA from these uteri was diluted (1:10; 1:100; 1:1000; and 1:10,000), or undiluted and used to construct a standard curve representing the natural log cDNA ng/mL versus Cycle Threshold (Ct) values for each gene product (r^2 = 0.98–0.99). The slopes for these relationships were: B-Actin, -3.32; Gsk3B, -4.65; Gys, -3.37 and Pyg, -4.5. The amount of each target gene amplification product (amplicon) was expressed in ng/mL and normalized by dividing by the corresponding amount of B-Actin gene product. Data were averaged by treatment group, and expressed in terms of a relative fold-difference compared to controls.

2.8. Western blot analysis (WBA)

A sample from each uterus (50 mg) was homogenized in RadioImmuno Precipitation Assay Buffer (Pierce Biotechnology, Rockford IL, USA). Protein concentration was determined using BioRad protein assay kits (500-0006; BioRad, Hercules, CA, USA). Approximately 36 μg of protein from each sample was separated into proteins of varying molecular weights by SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane using an Xcell II blot module (Invitrogen, Carlsbad, CA, USA), and blocked for 2 h in a 5% milk buffer containing Tween 40 (AC33414-2500; Fisher Scientific) to reduce non-specific binding.

Membranes were incubated with primary antibodies specific for total Gsk3B (9315; Cell Signaling Technology, Danvers, MA, USA), phospho-Gsk3B (p-Gsk3B, 9336S) or B-Actin (4967) at 1:1000 for 24 h at 4 °C. Blots were subsequently incubated with secondary antibody (anti-rabbit IgG conjugated to horseradish peroxidase; 7074; Cell Signaling Technology) at 1:2000 for 2 h at 4 °C. Blots were visualized by chemiluminescence using a luminol peroxidase kit (PI-34083, Pierce Chemical Company) and captured on photographic film (34092, Fischer Scientific).The relative amount of protein within the blots was quantified by densitometry using Un-Scan IT software (Silk Scientific Inc., Orem UT, USA), and expressed as the number of pixels per unit area, with all samples assayed in duplicate.

2.9. Statistical analysis

Only uteri from mink that survived to the completion of the experiment (Control = 6; E2 = 5, 4-OHE2 = 6 and 2-OHE2 = 4) were analyzed. Lack of tissue for some groups resulted in reduced sample sizes for WBA (4-OHE2 = 3; Control = 4). Comparisons between treatments and controls were made using one-way ANOVA, followed by Bonferroni's post test (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered significant at $P \leq 0.05$. Statistical differences between the endometrium and myometrium, and glandular and luminal epithelia for individual treatments were determined using unpaired Student's *t*-test ($P \leq 0.05$).

3. Results

Uterine weights of ovariectomized mink were increased approximately 4-fold by estradiol and 3-fold by 4-OHE2 and 2-OHE2 ($P \leq 0.05$) compared to controls, with no difference in uterine weights between 2-OHE2 and 4-OHE2 treated mink (Table 2). Endometrial and myometrial areas were increased 3- and 4-fold respectively, above controls by all three steroids (Table 2, Fig. 1; $P \leq 0.05$). The myometrial area was significantly greater than the endometrial area in control and all steroid-treated animals.

Glycogen concentrations in whole uterine homogenates were increased approximately 10-fold above controls by all three steroids (Table 3, $P \leq 0.05$). Total endometrial glycogen content, as determined with ImageJ, was increased 20-fold by 2-OHE2, 47-fold by

Table 2
Mean (± SEM) uterine weights and endometrial and myometrial areas of ovariectomized mink treated with estradiol, 4-hydroxycatecholestradiol (4-OHE2), 2-hydroxycatecholestradiol (2-OHE2), or as controls. Relative fold-increase above controls are given in parentheses.

Group	No. mink	Uterine weight (mg)	Endometrial area, (pixels × 10^4)	Myometrial area, (pixels × 10^4)
Control	6	322.23 ± 42.33[a]	3.68 ± 0.34[Aa]	6.41 ± 0.35[Ba]
Estradiol	5	1352.10 ± 145.47[b] (4)	11.07 ± 0.67[Ab] (3)	24.17 ± 1.26[Bb] (4)
4-OHE2	6	854.56 ± 101.51[c] (3)	9.86 ± 1.01[Ab] (3)	23.58 ± 1.66[Bb] (4)
2-OHE2	4	826.75 ± 107.39[c] (3)	9.82 ± 3.05[Ab] (3)	22.83 ± 1.60[Bb] (4)

[a–c]Within a column, means without a common superscript differ (P ≤ 0.05).
[A,B]Within a row, mean endometrial and myometrial values without a common superscript differ (P ≤ 0.05).

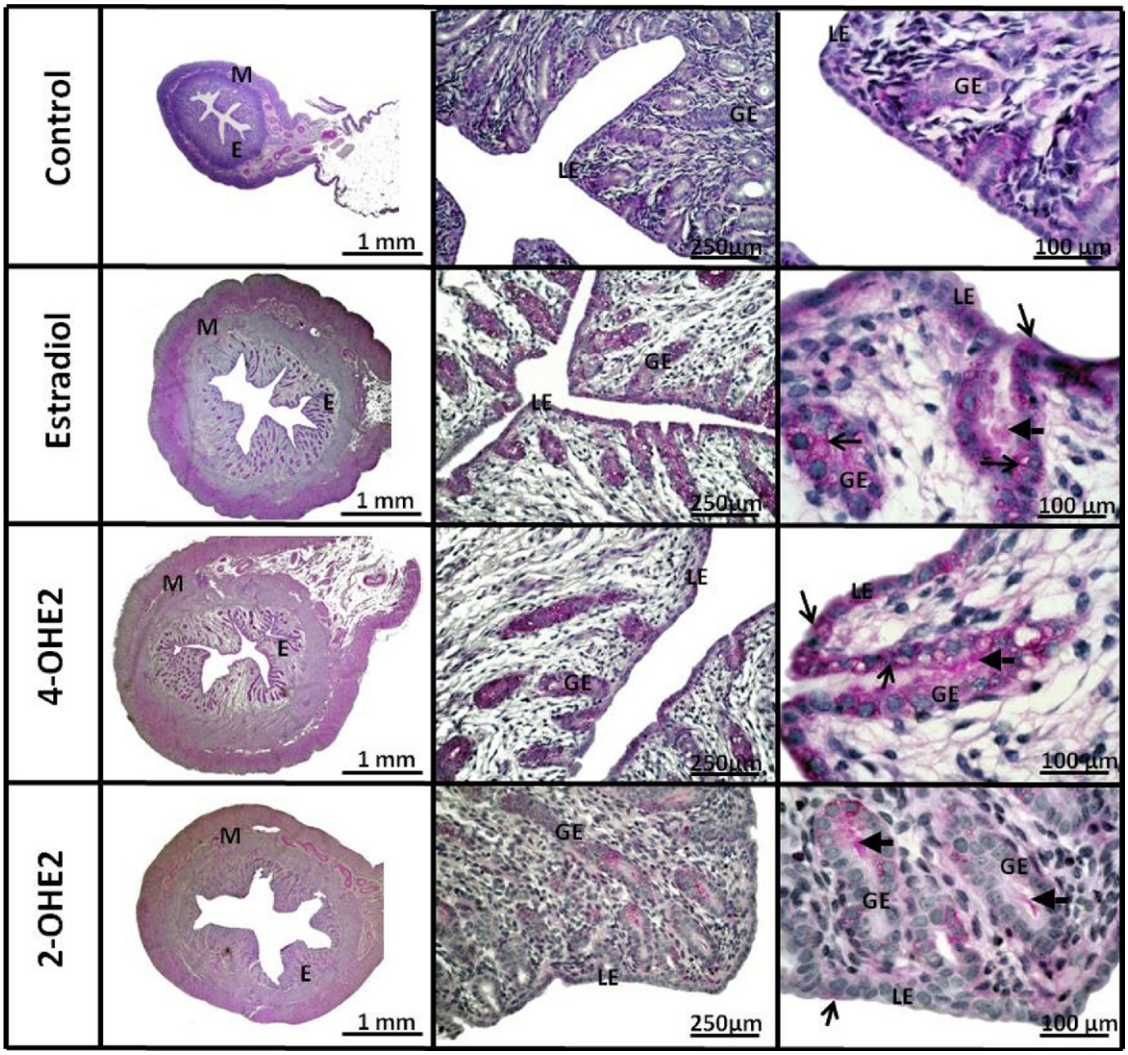

Fig. 1. Uterine cross-sectional images of mink treated with estradiol, 4-hydroxycatecholestradiol (4-OHE2), 2-hydroxycatecholestradiol (2-OHE2) or as controls. All sections (4 μm thick) were stained with PAS and counterstained with hematoxylin. Images were captured at 25× (left column), 400× (middle column) and 1000× (right column). Small arrows identify areas of positive PAS staining in uterine cells. Large arrows identify positive PAS staining within the glandular lumen. M, myometrium; E, endometrium; GE, glandular epithelium; LE, luminal epithelium.

Table 3
Mean ± SEM glycogen concentrations in whole uteri, and total glycogen content for endometrium and myometrium of ovariectomized mink treated with estradiol, 4-hydroxycatecholestradiol (4-OHE2), 2-hydroxycatecholestradiol (2-OHE2), or as controls. Relative fold-increase above controls are given in parentheses.

Group	No. mink	Glycogen (mg/g dry weight)	Endometrial glycogen (pixels × 10^3)	Myometrial glycogen (pixels × 10^3)
Control	6	4.58 ± 1.16^{aa}	0.09 ± 0.01^{Aa}	0.10 ± 0.02^{Aa}
Estradiol	5	43.79 ± 5.35^{b} (10)	5.68 ± 0.44^{Ab} (62)	8.33 ± 0.85^{Bb} (80)
4-OHE2	6	48.64 ± 4.02^{b} (10)	4.30 ± 0.40^{Ab} (47)	9.30 ± 0.80^{Bb} (90)
2-OHE2	4	41.36 ± 3.23^{b} (10)	1.85 ± 0.41^{Ac} (20)	11.0 ± 1.18^{Bb} (107)

[a–c]Within a column, means without a common superscript differ ($P \leq 0.05$).
[A,B]Within a row, mean endometrial and myometrial values without a common superscript differ ($P \leq 0.05$).

4-OHE2 and 62-fold by estradiol (Fig. 1, $P \leq 0.05$). Total myometrial glycogen content was increased 80-fold by estradiol, 90-fold by 4-OHE2, and 107-fold by 2-OHE2 ($P \leq 0.05$). The total glycogen content did not differ between the endometrium and myometrium in control mink, whereas all three steroids increased total glycogen content of the myometrium compared to the endometrium ($P \leq 0.05$). When normalized for differences in size (area) the percent glycogen content of the endometrium was greater than the myometrium in estradiol-treated mink (5.13 ± 0.35 vs 3.45 ± 0.27; $P \leq 0.05$), but not in controls (0.24 ± 0.026 vs 0.16 ± 0.022). The percent glycogen content was greater in the myometrium than endometrium in response to 2-OHE2 (4.83 ± 0.43 vs 1.88 ± 0.50; $P \leq 0.05$) and there was no difference in response to 4-OHE2 (4.36 ± 0.44 vs 3.94 ± 0.31).

The percent glycogen content of the glandular epithelia was increased 11-fold by 2-OHE2, 37-fold by 4-OHE2 and 47-fold by estradiol ($P \leq 0.05$) above controls (Fig. 1, Table 4). Percent glycogen content of the luminal epithelia was increased 3-fold by 2-OHE2, 13-fold by 4-OHE2 and 14-fold by estradiol ($P \leq 0.05$) above controls. The percent glycogen content did not differ between the glandular and luminal epithelia of control mink. Treatment with estradiol or 4-OHE2 resulted in a larger percent glycogen content in the glandular than luminal epithelia ($P \leq 0.05$).

Expression of Gsk3B mRNA by the mink uterus was reduced approximately 25% by estradiol and 2-OHE2 and 50% by 4-OHE2 ($P \leq 0.05$; Fig. 2). At the protein level, WBA revealed no difference in the amount of Gsk3B (total) in response to any treatment, nor between hormone treatments and controls (Fig. 3). Expression of p-Gsk3B (inactive) protein was barely detectable in ovariectomized untreated mink. Exogenous 2-OHE2 and estradiol increased production of p-Gsk3B protein 30 and 45-fold ($P \leq 0.05$), respectively, while 4-OHE2 increased production 5-fold, above controls ($P \leq 0.05$). Expression of Gys mRNA by the mink uterus was increased by both CEs ($P \leq 0.05$), but was unaffected by estradiol (Fig. 2). Uterine Pyg mRNA expression was reduced by all three steroids ($P \leq 0.05$).

4. Discussion

Growth of the mink uterus was increased approximately 4-fold by estradiol and 3-fold by 4-OHE2 and 2-OHE2 (Table 2, Fig.1). The myometrium was enlarged 4-fold and endometrium 3-fold by all three steroids. These responses may reflect the large dose of

Table 4
Mean ± SEM percent glycogen content for glandular and luminal epithelia of ovariectomized mink treated with estradiol, 4-hydroxycatechol estradiol (4-OHE2), 2-hydroxycatecholestradiol (2-OHE2), or as controls. Relative fold-increase above control values are given in parentheses.

Groups	No. mink	Glandular epithelia	Luminal epithelia
Control	6	0.45 ± 0.15^{Aaa}	0.50 ± 0.14^{Aa}
Estradiol	5	21.11 ± 4.14^{Ab} (47)	7.36 ± 0.85^{Bb} (14)
4-OHE2	6	16.57 ± 3.67^{Ab} (37)	6.67 ± 0.79^{Bb} (13)
2-OHE2	4	5.08 ± 2.75^{Ac} (11)	1.44 ± 0.28^{Ac} (3)

[a–c]Within a column, means with different superscripts differ ($P \leq 0.05$).
[A,B]Within a row, means with different superscripts differ ($P \leq 0.05$).

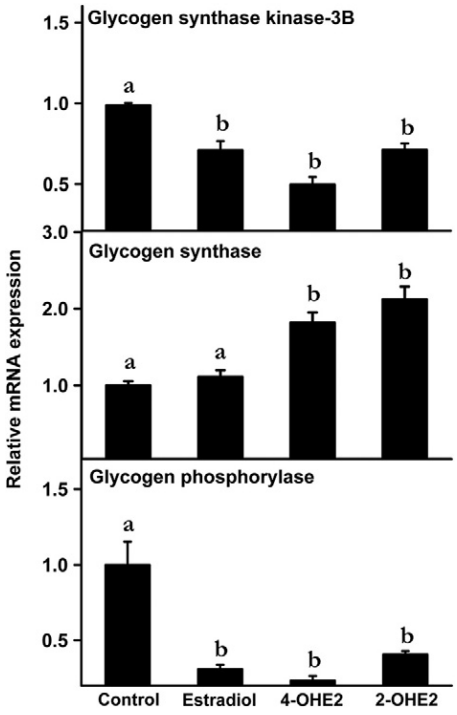

Fig. 2. Relative gene expression levels for glycogen synthase kinase-3B, glycogen synthase and glycogen phosphorylase (mean ± SEM) by the uteri of ovariectomized mink treated with estradiol (N = 5), 4-hydroxycatecholestradiol (4-OHE2; N = 6), 2-hydroxycatecholestradiol (2-OHE2; N = 4) or as controls (N = 6).
[a,b]Groups without a common letter differ ($P < 0.05$).

hormones used, but could be species specific. In the immature rat, estradiol and 4-OHE2 at 10 ng/d, produced equivalent uterine weight gains, whereas 1000 to 10,000 ng/d of 2-OHE2 were required to produce comparable weights [31]. Treatment of CD-1 mice with these steroids at 2 μg/d during the first 5 d of life resulted in uterine weight gains of 133% (estradiol), 213% (4-OHE2) and 107% (2-OHE2) [32].

Whole uterine glycogen concentrations, as well as total endometrial and myometrial glycogen content, were significantly increased by estradiol and both catecholestrogens (Table 3, Fig. 1). Although total glycogen reserves were greater in myometrium than endometrium, this appeared to be due in part, to the larger size of the myometrium (Table 2, Fig.1). The percent glycogen content of the glandular epithelia exceeded that of the luminal epithelia, in response to estradiol and 4-OHE2 ($P \leq 0.05$), but not 2-OHE2 (Table 4, Fig. 1). These findings agreed with those reported for other species, showing that exogenous estradiol stimulates uterine glycogen synthesis [18–21], and that 4-OHE2 normally has greater uterotrophic potency than 2-OHE2 (22–24). Large glycogen deposits within the glandular epithelia has been observed by others [5–7], and strengthens the hypothesis that glycogen is an important product of endometrial glands.

Expression of the Gsk3B gene by the mink uterus was reduced by all three steroids (Fig. 2), which could have contributed to increased glycogen synthesis. And yet, our WBA revealed no difference in Gsk3B (active) protein expression between hormone treatments and controls (Fig. 3). It is not known if Gsk3B expression differs between mink uterine endometrium and myometrium, or between glandular and luminal epithelia. If such differences exist, they could have been masked by our analyses of whole-organ homogenates. In support of this hypothesis, Gunin et al, [33–35] demonstrated Gsk3B protein immunostaining in mouse uterine luminal and glandular epithelia, but not stroma. Furthermore, treatment with estradiol resulted in a greater level of total Gsk3B immunostaining than in controls. Salameh et al [36] showed that the highest human uterine Gsk3B levels occurred in the endometrium, especially the glandular epithelia. We concluded that, although estradiol and both catecholestrogens reduced Gsk3B gene expression, they had no effect on Gsk3B protein formation.

Uterine expression of p-Gsk3B in control mink was barely detectible (Fig. 3). Exogenous 2-OHE2 and estradiol increased production of the protein 30 and 45-fold respectively, whereas 4-OHE2 increased production only 5-fold above controls. It was surprising that 2-OHE2, considered to be less estrogenic than 4-OHE2,

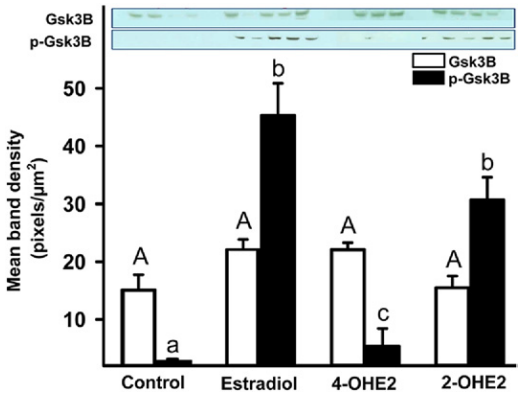

Fig. 3. Western blot analyses (WBA) for total glycogen synthase kinase-3B (Gsk3B) and p-Gsk3B (Gsk3B) proteins (mean ± SEM) expressed by the uteri of ovariectomized mink treated with estradiol (N = 5), 4-hydroxycatecholestradiol (4-OHE2; N = 3), 2-hydroxycatecholestradiol (2-OHE2; N = 4) or as controls (N = 4).
[a–c]Within a protein, groups without a common letter differ ($P < 0.05$).

had a greater effect on Gsk3B phosphorylation; this could represent a specific role for 2-OHE2 in mink uterine glycogen metabolism. Low p-Gsk3B protein expression by the uterus of control mink agreed with the findings of Chen et al [16], who reported that ovariectomized CD-1 mice failed to produce p-Gsk3B, whereas treatment with estradiol increased production of the protein within 2 h. Wang et al [37], using the ovariectomized cyclin D1 null mouse, could only detect p-Gsk3B at levels slightly above background in the uterus, whereas treatment with estradiol, produced a very strong signal for the protein within 5 h. Sanz et al [38] administered estradiol to ovariectomized rats, which resulted in a sustained phosphorylation of Gsk3B in the prefrontal cortex. Salameh et al [36] showed that although Gsk3B mRNA and total Gsk3B protein expression did not change throughout the human menstrual cycle, expression of p-Gsk3B increased more than 5-fold during the secretory phase, peaking after ovulation on d 17, just prior to the expected time of implantation. We concluded that increased glycogen accumulation in the mink uterus was due in part, to the phosphorylation and inactivation Gsk3B, in response to estradiol and catecholestrogens, especially 2-OHE2.

Expression of Gys mRNA by the mink uterus was increased by 2-OHE2 and 4-OHE2 ($P \leq 0.05$) but interestingly, was unaffected by estradiol (Fig. 2). To the best of our knowledge, all previous investigations on the effects of estrogens on uterine Gys have focused on enzyme activity and not gene expression [39–41]. We concluded that estradiol stimulated uterine glycogen synthesis by enhancing Gys activity, whereas Gys gene expression was increased by catecholestrogens. Thus, the concomitant actions of estradiol and catecholestrogens on the uterus may be requisite for optimal glycogen synthesis in the mink.

Mink uterine Pyg mRNA expression was reduced by estradiol, 2-OHE2, and 4-OHE2 (Fig. 2; $P \leq 0.05$). We are unaware of any published data on uterine Pyg gene expression, whereas there are numerous reports on Pyg activities [40,41]. Nevertheless, our findings agreed with those for the human, where uterine Pyg activity was lowest during the proliferative phase when estrogen concentrations were high [40,41]. In ovariectomized rats, a single sc injection of estradiol reduced uterine Pyg activity at 12 h, which gradually increased from 48 to 96 h [39]. It is likely that reduced Pyg mRNA expression in the mink uterus resulted in decreased p-Pyg protein expression, contributing to greater glycogen reserves. However, because commercial antibodies were unavailable for WBA of Pyg and p-Pyg, we were unable to make this determination.

We suspected that some conversion of exogenous estradiol to catecholestrogens would have occurred in the mink uterus. However, if this happened, the amounts were either very small and/or were catabolized at a rate that prevented any observable effects. Regardless, we have demonstrated expression of the CYP1B1 gene, that codes for the enzyme that produces 4-OHE2 [22,32], by the mink uterus and shown that exogenous estradiol doubles expression of the gene (unpublished data; [42]). These findings support those of Paria et al [24] who showed that treatment of ovariectomized mice with estradiol stimulated uterine 2, 4 hydroxylase activity. Moreover, 4-hydroxylase activity increased during the afternoon of Day 4, just prior to implantation, then declined on Day 5. They proposed that the increase in 4 hydroxylase activity might be due to the increase in preimplantation ovarian estrogen secretion that takes place around noon on Day 4. Similarly in the mink, we envisioned that catecholestrogen production by the uterus may increase during proestrous, mating and implantation when circulating estradiol concentrations were elevated [43–45].

It is unresolved whether uterine glycogen reserves are utilized only after glycogen is catabolized to glucose, or if in addition, embryos take up glycogen from the uterine histotroph by endocytosis. Preimplantation embryos of many species are unable to metabolize glucose until the early blastocyst stage, co-incident with differentiation of the epithelial trophectoderm [46–48]. At that time, embryos switch from oxidizing pyruvate and lactate to glucose metabolism, which appeared to be essential for further development. During blastocyst activation and implantation, uterine histotroph production increased and the trophectoderm took up macromolecules, apparently including glycogen [6,49–51]. We occasionally observed positive PAS staining within the lumens of the mink endometrial glands (Fig. 1); we inferred that glycogen was being secreted into the uterine lumen. The trophoblast of carnivores becomes extremely phagocytic as implantation progresses and the trophectoderm is capable of engulfing particles as large as whole erythrocytes [52]. It should not be surprising that embryos may derive glucose by direct uptake of the monosaccharide as well as through phagocytosis of glycogen from the uterine histotroph.

In summary, we concluded that glycogen, synthesized in response to estradiol, was an important nutrient produced by the uterine endometrial glands of mink.

Since 4-OHE2 and 2-OHE2 but not estradiol increased *Gys* gene expression, we inferred that the effects of estradiol on uterine glycogen synthesis were mediated in part through catecholestrogens. Although circulating estradiol concentrations in mink declined during estrus and pregnancy, their concentrations were still much higher than during the anestrous. As a consequence, we propose that estradiol and catecholestrogens will continue to promote uterine glycogen synthesis during these intervals. In that regard, such a mechanism could teleologically serve to divert circulating glucose to the uterus for local storage and use during embryo development, implantation, and perhaps pregnancy.

Acknowledgments

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Sequencing of the Aleutian Mink Disease Virus – A Preliminary Report

Hossain Farid (June 2011)

Where did this virus come from? This is often the first question a mink rancher would ask when his or her clean herd becomes infected with the Aleutian mink disease virus (AMDV). There are instances where ranchers, particularly in areas with a high density of mink farms, have followed recommended biosecurity measures (or they thought they had), yet mysteriously became infected with the virus. The virus may come from other infected ranches, wildlife populations or the soil on the ranch where it could have been hiding for years. Knowing where the virus came from would help ranchers block the entry route of the virus. Knowledge of the sources of infection would be a key step in the fight against the AD virus infection.

These days, it would be possible to pin-point the source of the virus. All that is needed is the sequence of the virus on the infected ranch, and a database containing sequences of other viruses in ranched mink and wild animal populations. Sequence of the virus on a ranch could be compared with those in the database to identify the source of the new outbreak. This is the main reason that we are sequencing the AD virus in Nova Scotia. The AD virus is known to be highly variable because of its high mutation rate. This high degree of variability would work as a >DNA tag= for accurate identification of the source when AD positive mink appear on clean ranches.

High levels of variability of the viral genome have some downfalls as well. It may reduce the accuracy of virus detection by the polymerase chain reaction (PCR). Every PCR test requires two primers, which work as tags or addresses for the test to be performed correctly. Any mismatch between the primer sequences and the virus causes the failure of the test, i.e., would increase the likelihood of false negative. Sequencing of a large portion of the viral genome is necessary to identify regions that are similar among all viral types in Nova Scotia, helping us to develop PCR tests that can be used universally. This is the second reason that we are sequencing a large segment of the AD virus in Nova Scotia.

All mink ranchers know that some AMDV types are nasty and cause high mortality, while others are less dangerous. All the information on the pathogenicity of a virus is present in its DNA sequence. Scientists have already identified regions of the viral genome that dictate, to a great extent, the degree of pathogenicity of the AMDV strains. A database of viral sequences in a region can be linked with the mortality rate caused by a specific virus strain, as well as with the published sequences of the viruses with know pathogenicity. It is thus theoretically possible to distinguish between the good, the bad and the ugly, using the genome sequence database. This is the third reason that we sequence the AD virus in Nova Scotia.

The AMDV genome is 4,801nucleotide long. This is a very small genome. To identify ADV types in Nova Scotia, DNA was extracted from frozen spleen tissues of mink that were tested positive by Counter-immunoelectrophoresis (CIEP). A 4410 nucleotide long fragment of the viral genome, from nucleotides 206 to 4616, was amplified by PCR. This fragment, which covers 91.9% of the viral genome, contains the segment of the viral genome that is required by the virus for infecting the mink and replication. PCR products of 104 samples from 15 ranches have so far been sequenced. Our database already contains a few times greater the volume of viral genome sequence data than all the published information.

Viral types

The preliminary results showed that more than 70% of the samples were different from each other by at least one nucleotide. This high degree of variability is in agreement with the high mutation rate of the virus. To date, we have found two groups, 8 types and many sub-types in Nova Scotia. One of the groups (Group 2) is 27 nucleotides shorter than the other group. More than one viral type circulated on most

ranches, particularly on those that have been infected for many years. Viruses on each newly infected ranch were similar to each other. Although it is too early to say, but it seems that the number of different viral types and sub-types is a measure of the history of infection of a ranch. Some animals were infected with more than one viral type or sub-type. The effects of this phenomenon on animals' response to infection are not known.

Comparison between virus types and published information

It is difficult to compare the viral types in Nova Scotia with viral strains in other parts of the world whose DNA sequences have been published, mainly because very few have complete or almost complete published sequences. The following Table shows the number of sequences overlapped between ours and published sequences. The strain 'G' is the only one that has been completely sequenced. Other strains have short published sequences.

The following table shows percentage similarities between one virus from Nova Scotia (Group 1) and 11 published strains. Utha-1, Danish-K and TR are highly pathogenic, Pullman is moderately pathogenic and 'G' is non-pathogenic. Far East strain is from Russia and its virulence has not been reported. Although it is early to say, but all Nova Scotia types seems to be different from published strains, particularly the TR, Pullman, Danish-K and United.

Strain	G	SL3	Utah I	Utah I	Utah I	Danish-ZK8	Danish-K	United	TR	Pullman	Far East
Overlap	4410	4359	4402	3454	2003	3455	2003	2003	1944	1944	1941
% similarity	97.53	97.23	96.10	96.73	96.56	96.79	88.02	93.21	96.77	91.31	94.49

Viruses in Group 2 seem to be related to the TR strain, which is a pathogenic strain that caused an outbreak of AD in Utah in mid 1990s (both types are missing the same 27 nucleotides, which cannot happen very frequently). This, however, does not mean that Nova Scotia viruses in group 2 are pathogenic.•

HEALTH

Infuenza In Mink

John R. Gorham, D.V.M.

There are several influenza viruses, human, swine, horse and avian. You guessed it. Mink are susceptible to all of them.

The first reported outbreak occurred on a Japanese mink farm when young mink were found to be infected with a human flu virus. This suggested that a mink farmer with the flu transmitted the virus to his mink by coughing or sneezing.

In 1979, the Japanese intranasally infected mink and confirmed that mink were

susceptible to several strains of influenza virus. It is obvious that if the flu virus can easily be transmitted from man to mink, virus can also be transmitted from mink to mink. This means that future influenza outbreaks on farms will occur.

The first outbreaks that really got the mink farmers' attention were the influenza outbreaks that appeared along the coast of southern Sweden in 1984. Mink showed symptoms of a respiratory disease---lack of appetite, depression, squinty crusty eyes, broncho-pneumonia and, in some cases, death. Remains of dead wild birds that had been eaten by mink were picked up around the mink pens. This led to a diagnosis of avian influenza as the cause of the outbreaks. In 2006, another Swedish outbreak of mink influenza occurred in which wild birds also were believed to be the source of the virus.

Uncooked pork byproducts obtained from local slaughter houses were the source of a 2007 Canadian mink outbreak. A swine influenza virus was isolated. According to the Avian Flu Diary, the prevalence of influenza in Canadian farmed mink and in wild mink is unknown.

Anytime a virus-like influenza selects and infects a new species, it is of real interest. For example, in 2009 when a new strain of human influenza virus infected mink on 11 farms in the Holstebro area of Denmark, it was determined that this virus had previously infected people during the four years before the mink outbreaks.

Henrik Jorgensen of Denmark's National Veterinary Institute says, "It is a situation that creates some concern because we can see that the virus originates in humans. With this background, we conclude that there is an increased risk that it also can infect back to people."

This is apparently the first recorded case seen in the world where a human influenza infected mink. Jorgensen continues, "We do not know how the influenza virus entered the farms and we never got it solved. But it probably is a single factor because Holstebro is a geographically limited area. It may be via food or by an individual who has spent time here on the farms."

The transmission of human flu, avian and swine flu viruses to mink is of real concern because the virus has the potential of establishing itself on a farm and spreading to other mink farms. We really do not need another mink disease.

At this writing (June 2010), outbreaks of influenza in farm raised mink in the United States have not been reported. No flu vaccines have been developed for mink and effective treatment is questionable. Laboratory tests are available to confirm cases of mink flu.

Ferrets were first experimentally infected with a human influenza virus in the 1920s and many times since then. Recently, pet ferrets (a species related to mink) in Portland and Roseburg, Oregon were infected with the H1N1 human influenza virus. Family members had flu-like symptoms and some ferrets had a fever, nasal exudates and respiratory signs.

Naturally occurring influenza A virus (H1N2) infection in a US Midwest mink ranch

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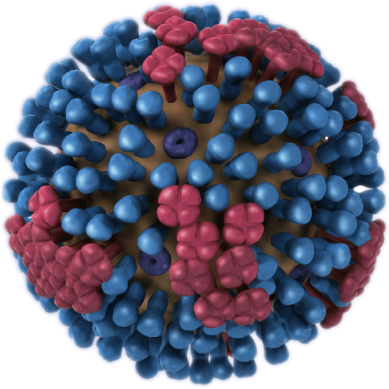

ABSTRACT

Influenza A virus (IAV) causes acute respiratory disease in humans and a variety of animal species. The virus tends to remain within the species of origin; nonetheless, naturally occurring cross-species transmission of IAV has been periodically documented. Recently multiple cross-species transmissions of IAV have been reported from companion animals and captive wild animals, neither of which is historically considered as natural hosts of IAV. In the fall of 2010, a 15,000-head mink farm in the Midwest USA experienced persistent severe respiratory distress and nose/mouth bleeding, with death loss of approximately 10 animals per day. Six dead mink (Mustela vison) at 6 months of age were submitted to the Iowa State University Veterinary Diagnostic Laboratory for diagnostic investigation. Gross and microscopic examinations revealed that all 6 mink had hemorrhagic bronchointerstitial pneumonia. Hemolytic E. coli was isolated from lungs, probably accounting for hemorrhagic pneumonia. All animals were tested negative for canine distemper virus and Aleutian disease virus. Interestingly, IAV of H1N2 subtype, which contained the matrix gene of swine lineage, was detected in the lungs. Serological follow-up on mink remained in the ranch until pelting also confirmed that the ranch had been exposed to IAV. The case study suggests that IAV should be included in the differential diagnosis when mink experience unresponsive or epidemics of respiratory disease. Since the source of IAV appeared to be uncooked turkey meat, feeding animals fully cooked ration should be considered as a preventive measure.

Influenza A virus (IAV) causes acute respiratory disease with fever, commonly referred to as “Flu”, in humans and a variety of animal species.25 In animals, IAV infection has been commonly identified in pigs, horses, sea mammals and poultry. The virus and disease were typically named after its host of isolation, e.g., swine influenza (“swine flu”) virus (SIV), equine influenza virus, and avian influenza (“bird flu”) virus (AIV). Each of these viruses tends to remain within the species of origin and maintain distinct genetic profiles from each other. Nonetheless, naturally occurring cross-species transmission of IAV has been periodically documented.3,4,8,17 Since IAV can be passed between humans and animals1,9,24, the virus is a zoonotic pathogen. Most of the cross-species transmission events are self-limiting without any further implication. In some cases such an event introduced a new IAV strain to a new population through adaptation or reassortment process.1,7,13,15,22,23,27 Recently, multiple cross-species transmissions of IAV have been reported from companion animals4,12,16,17,18,19 and captive wild animals5,6,10,14,21, neither of which is historically considered as natural hosts of IAV. While exact mechanisms for successful cross-species transmission require further study, such an event brings a new perspective and challenge to the influenza ecology, which should be taken into consideration when dealing with respiratory problem in those animal species.

In mid October of 2010, ranch-raised mink in the Midwest USA experienced persistent severe respiratory distress, with death loss of approximately 10 animals per day. The farm had 15,000 mink, of which 3500 were breeding females and the rest kits and growers. No other animals except farm dogs were on the ranch. The mink were fed poultry meat. Affected animals were lethargic, had mucoid and watery discharge

from the eyes, as well as hemorrhage from the mouth and nose. The most notable clinical sign was severe cough which could be heard throughout the ranch. The clinical problem affected both adults and kits, with affected mink spread randomly across the ranch. The death loss was also widespread across the ranch during the outbreak, although it was usually concentrated in a specific area of the farm on any particular day.

Six dead 6-month-old mink (Mustela vison) nearing maturity were received by the Iowa State University Veterinary Diagnostic Laboratory for diagnostic investigation. Gross examination revealed all 6 mink had locally extensive or diffuse hemorrhagic pneumonia. No gross lesions were apparent in other body systems. Microscopically, lungs had severe diffuse congestion with locally extensive mixed bronchointerstitial pneumonia. Terminal airways had necrotizing bronchiolitis, plugs of mixed inflammatory and necrotic debris, and had moderate nonsuppurative peribronchiolar and perivascular cuffs.

Hemolytic Escherichia coli was isolated from lungs. No nucleic acid of Aleutian disease virus was detected in spleens. Lungs, brains and bladder swabs were tested negative for canine distemper virus by a fluorescent antibody test of frozen tissue sections and/or PCR assay. Because lung lesions were suggestive of epitheliotropic viral insult, the samples were tested for IAV by immunohistochemical staining and PCR assay. Virus antigen was detected within lesions. Viral nucleic acid was also detected. Hemagglutinin (H) and neuraminidase (N) genes of the virus was subtyped H1N2. By sequence analysis, the virus appeared to be of swine origin as its matrix (M) gene showed close genetic relatedness to the M gene of US endemic SIV (Fig. 1). Approximately 2 months after the initial outbreak, serum samples were randomly collected from 20 mink during pelting and tested for IAV antibody. All except one animal were strongly seropositive for IAV on a commercial ELISA2, further supporting that the mink from this ranch were exposed to IAV. Hemagglutination inhibition (HI) tests against a battery of IAV with H1 subtype demonstrated that the animals had significant levels of HI antibody (≥1:40) against H1 of cluster23 and no HI activity against H1 viruses of , or cluster (Fig. 2).

The outbreak of severe hemorrhagic pneumonia in the index mink ranch was due to concurrent infection with IAV and E. coli. While the severity and persistence of clinical disease was due to IAV infection, hemorrhagic pneumonia with a high mortality was more likely attributed to secondary bacterial infection; high mortality is not a common feature of IAV infection in mammals without bacterial co-infection of lungs. Similar clinical presentation was observed with IAV infection in racing dogs when the animals were concurrently or subsequently infected with bacteria.26 The clinical outcome, particularly mortality, perhaps could be mitigated with use of antibiotics in acute outbreaks of influenza.

The mink were not fed pork byproducts and there were no swine herds nearby; therefore, it was unexpected to identify IAV of swine origin. Interestingly the owner of the mink ranch mentioned feeding of animals with turkey meat when chicken meat could not be supplied. Although a formal epidemiological study was not conducted, it is then logical to speculate that the source of IAV was turkey since cross-species transmission of SIV, particularly H1N2 and H3N2, to turkeys commonly occurs, particularly after triple reassortant H3N2 IAV emerged in US swine.3, 20 Since the source of meat may have a role in bacterial and/or viral contamination in ration, the clinical episode in the mink ranch emphasizes the importance of careful screening source of meat as a preventive measure when mink are fed an uncooked ration. As a better intervention, feeding animals with fully cooked ration should be considered.

Mink, as well as some small mammals such as ferrets, guinea pigs and mice, are known to be susceptible to IAV and have been frequently used in laboratories to study IAV derived from humans and other animal species.25 Hence, it is not totally unexpected that mink or ferret fur farms undergo influenza outbreak when animals are fed on IAV-contaminated meats. Indeed there is a previous report of naturally occurring H3N2 IAV infection in a mink farm in Canada where animals were fed uncooked pig meat byproducts.6 Therefore, veterinary professionals and mink producers should include IAV in the differential diagnosis when animals experience unresponsive or epidemics of respiratory disease.

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