Antiretroviral efficacy of a 98% solution of glycerol or ethylene oxide for inactivation of feline leukemia virus in bone

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Objective—To determine whether infectious retrovirus was inactivated in bones from FeLV-infected cats after ethylene oxide (ETO) sterilization or preservation in a 98% solution of glycerol in an in vitro cell culture system.

Sample Population—Metatarsal bones obtained from 5 FeLV-infected cats and cultured with feline fibroblast cells.

Procedure—Metatarsal bones were treated with 100% ETO, a 98% solution of glycerol, or left untreated. Twenty-five flasks of feline fibroblast cells were assigned to 5 groups: negative control, positive control, ETO-treated bone, glycerol-treated bone, and untreated bone with 5 replicates/group for 4 passages. Media and cell samples were harvested from every flask at each passage to measure FeLV p27 antigen and the number of copies of provirus per 100 ng of DNA, respectively.

Results—All negative control and ETO-treated group replicates were negative for FeLV p27 antigen and provirus throughout the study. All positive control group replicates were positive for FeLV p27 antigen and provirus at passages 1 to 4. Untreated bone group replicates were positive for FeLV p27 antigen at passages 3 and 4 and provirus beginning at passage 2. Glycerol-treated group replicates had delayed cell replication and were negative for FeLV p27 antigen and provirus at passages 1 to 4 and 2 to 4, respectively.

Conclusions and Clinical Relevance—Ethylene oxide sterilization of bone from FeLV-infected cats appeared to abrogate transmission of infectious retrovirus and effectively sterilized bone allografts.

Impact for Human Medicine—Additional studies to confirm effectiveness of ETO treatment of allograft tissues for prevention of pathogen transmission via transplantation are warranted. (*Am J Vet Res* 2004;65:436–439)

Transplantation of soft tissue and bone allografts raises concerns regarding possible transmission of infectious diseases. Although the likelihood of transmitting a bacterial infection may be markedly reduced through sterile harvesting and antimicrobial treatment of grafts, these procedures would not affect viral infectivity. Transmission of **human immunodeficiency virus (HIV)** to human recipients of bone and soft tissue allografts has been reported.[1-6] Efforts to prevent HIV transmission following transplantation of allograft tissues have mainly relied on obtaining a thorough donor history and blood screening.[7-9]

Results of previous studies[10-13] indicate that a retrovirus similar to HIV was efficiently transmitted through transplantation of FeLV-infected bone and connective tissue allografts exposed to a variety of processing and sterilization techniques. The purpose of the study reported here was to determine whether infectious retrovirus was inactivated in bone obtained from systemically FeLV-infected cats after **ethylene oxide (ETO)** sterilization or preservation in a 98% solution of glycerol in an in vitro cell culture system. We hypothesized that treatment of FeLV-infected bone with 100% ETO would prevent, whereas preservation with a 98% solution of glycerol would permit, transmission of the retrovirus to susceptible cells.

Materials and Methods

Bone processing—Four weeks prior to cell culture inoculation, 3 metacarpal bones from each of 5 FeLV-infected (Rickard strain), **specific pathogen-free (SPF)** cats from a previous study[13] were aseptically harvested and stored fresh-frozen at –70°C in individual sterile plastic conical tubes.

The 3 metacarpal bones from each FeLV-infected donor cat were allocated to the following groups: ETO-treated, glycerol-treated, or untreated bones. The ETO-treated bones were separately double-wrapped with heat-sealed plastic, sterilized with 100% ETO, aerated at 50°C for 12 hours (relative humidity, 30%), and refrozen at –70°C. Glycerol-treated bones were individually placed into conical centrifuge tubes containing a 98% solution of glycerol and stored in a dark environment at 22°C for 4 weeks.[12] Bones for the untreated group were stored in separate sterile plastic conical tubes at –70°C.

Cell culture—Twenty-five flasks of **feline fibroblast cells (FEA cells)** were allocated to 5 groups: negative-control, positive-control, ETO-treated bone, glycerol-treated bone, and untreated bone with 5 replicates/group for 4 passages. Confluent cells were trypsinized, resuspended at a 1:3 dilution, and introduced into separate sterile flasks containing 10 mL of media on the day before test samples were added. The FEA cell media consisted of Dulbecco's modified eagle medium with 15% fetal bovine serum, 2% glutamine, 2% sodium bicarbonate, 1% sodium pyruvate, gen-

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tamicin sulphate (10 µg/mL), and enrofloxacin (10 µg/mL). Media and cells from stock cultures were confirmed negative for FeLV p27 antigen and provirus, respectively. Media was aspirated from the adherent FEA cells, 10 mL of media containing diethylaminoethyl dextran (0.03 mg/mL) was added for 30 minutes, then replaced with 10 mL of FEA cell media when test samples were added. Negative and positive control groups consisted of an additional 0.5 mL of media or supernatant fluid from a productively FeLV-infected cell line (FL-74 cells), respectively. Each treated or untreated bone was individually minced with Lembert rongeurs, and 250 mg was immediately introduced into separate FEA cell culture flasks. Flasks were placed into a 37°C, 5% carbon dioxide, humidified incubator. Cell cultures were permitted to grow until they reached confluence, seen as a homogeneous layer of FEA cells covering the dependent wall of the flask when viewed with an inverted light microscope. Media from each flask was individually saved, cells were trypsinized, pelleted by centrifugation, supernatant fluid was discarded, and a 1:15 dilution of resuspended cells was introduced into new flasks containing 10 mL of media. Remaining cells from each flask were saved for DNA extraction and quantification of FeLV provirus. This process was repeated for a total of 4 passages to promote viral amplification.

Quantitation of FeLV p27 antigen—An ELISA kit[a] was used to detect FeLV p27 antigen in culture media samples. The **optical density (OD)** was measured at 630 nm by use of a densitometer.[b] The **standardized optical density ratio (SODR)** was calculated by use of the following formula:

$$\frac{(\text{OD of sample}) - (\text{OD of negative control sample})}{(\text{OD of positive control sample}) - (\text{OD of negative control sample})}$$

The SODR corrects for interassay variations by standardizing the OD of the sample against manufacturer-supplied positive and negative controls. Calculated SODRs ≥ 0.1 were considered positive for FeLV p27 antigen.[12-14]

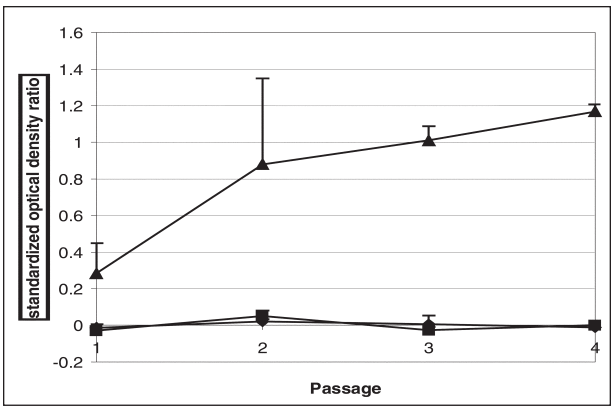

Figure 1—Standardized optical density ratio values for FeLV p27 antigen in media from feline fibroblast cells cultured with ethylene oxide-treated (diamonds), glycerol-treated (squares), or untreated (triangles) bones obtained from systemically FeLV-infected cats for 4 passages.

DNA extraction, quantification, and digestion—The DNA was extracted from FEA cells by use of a commercially available kit,[c] quantified with a DNA flourometer,[d] linearized by use of the restriction endonuclease EcoRI, and stored at 4°C in sterile microcentrifuge tubes.

Nucleic acid amplification testing for FeLV provirus—A unique 65-bp fragment of exogenous FeLV was amplified by real-time quantitative polymerase chain reaction assay[e] from 100 ng of EcoRI digested DNA extracted from FEA cell samples. Primers[f] and an internal sequence-specific fluorescent probe[g] for the U3 region of exogenous FeLV were used.[12,13,15] Serial 10-fold calibration standard dilutions and negative controls were performed in parallel with test samples. Assays for each specimen were performed in triplicate with results reported as copy number/100 ng of DNA. Results ≥ 23 copies/100 ng of DNA were considered positive for FeLV provirus.[12,13,16-18] Although FeLV provirus was amplified by the polymerase chain reaction assay, FeLV virus may have been replicated in the cell culture system.

Statistical analyses—Results for FeLV p27 antigen and provirus were assigned a positive or negative value and compared by use of a 2-tailed Fisher exact test.[h] Differences were considered significant at $P < 0.05$.

Results

FeLV p27 antigen—Results for all media samples in negative control, ETO-treated, and glycerol-treated groups were negative for FeLV p27 antigen throughout the entire study. Results for all samples in positive control and untreated bone groups were positive for antigen at passages 1 to 4 and 3 to 4, respectively (**Fig 1**). Significant differences in the SODRs ($P < 0.05$) between positive control and untreated versus negative control, ETO-treated, and glycerol-treated groups were observed at all 4 passages.

FeLV provirus or virus—Results for all negative control and ETO-treated group cell culture samples were negative for FeLV proviral DNA throughout the entire study. Results for all positive control and untreated bone group specimens were positive for FeLV provirus or virus starting at passage 1. Results for 1 glycerol-treated group sample was positive for FeLV provirus at the first passage then negative at passages 2 to 4 (**Table 1**). Significant differences in the number of provirus copies per 100 ng of DNA ($P < 0.05$) between positive control and untreated versus

Table 1—Feline leukemia virus proviral copy numbers determined by quantitative polymerase chain reaction assay in DNA extracted from feline embryonic fibroblast cells cultured with untreated, ethylene oxide (ETO)-treated, or glycerol-treated bone from each donor cat at each passage

	Passage 1			Passage 2			Passage 3			Passage 4		
Donor cat	**ETO**	**Glycerol**	**Untreated**	**ETO**	**Glycerol**	**Untreated**	**ETO**	**Glycerol**	**Untreated**	**ETO**	**Glycerol**	**Untreated**
118	0.55	1.06	118	0.03	0.12	431,740	0.10	0.72	203,775	0.24	0.89	105,207
120	0.06	124.99	487	0.13	14.85	291,549	0.72	1.84	1,511	0.48	0.40	118,846
121	0.01	0.67	2	0.70	0.20	121	0.01	0.25	171,062	0.01	0.01	130,364
127	0.01	5.09	12,384	0.01	0.21	360,277	0.01	0.33	2,155	0.34	0.67	2,689
128	0.02	5.47	165	0.08	0.22	181,970	0.03	0.01	2,088	0.51	0.67	69,988
Mean	0.13	27.46	2,631	0.19	3.12	253,131	0.17	0.63	76,118	0.32	0.53	85,419
SD	0.24	54.57	5,455	0.29	6.56	168,771	0.31	0.72	102,259	0.20	0.34	51,506

Results ≥ 23 copies/100 ng DNA were considered positive for FeLV provirus.

negative control, ETO-treated, and glycerol-treated groups were observed at passages 1 to 4.

Discussion

Feline leukemia virus is a safe and effective in vitro and in vivo model for investigation of antiretroviral treatments including those aimed at preventing transmission from infected bone and connective tissues.[10-14,19-25] Although the lentivirus **simian immunodeficiency virus (SIV)** is more closely related to HIV than FeLV, higher purchase, housing, and maintenance costs, as well as risks of serious injury and disease transmission to investigators, preclude the use of this model for most studies.[26] Another lentivirus model, FIV, has the limitations of a restricted susceptible host cell range (lymphocytes, monocytes and macrophages, astrocytes, microglial cells, and endothelial cells) and lack of documented transmission from bone or connective tissues.[27-29] In contrast, FeLV infects a broad array of host cell lines (neutrophils, platelets, lymphocytes, monocytes and macrophages, epithelial cells, and mesenchymal cells), rapidly induces a high virus titer, and is readily transmitted from bone and connective tissues in vitro and in vivo.[10-13,30-36] Availability, safety, convenient size, and cost-effectiveness make the FeLV-cat model attractive.

Sterilization with ETO appeared promising for inactivation of FeLV in a previously reported in vivo study[12] in which 5 recipients of systemically FeLV-infected, ETO-sterilized bone allografts remained negative for FeLV antigen and provirus. Reduced viral infectivity from ETO treatment, as well as an effective host immune response, may have contributed to the lack of observed retroviral transmission in vivo. Amplification of FeLV provirus from 3 of 5 ETO-treated donor bone grafts raised concerns over the possible presence of residual infectious retrovirus.[12] However, the small FeLV amplicon may have been derived from fragments of noninfectious, degraded DNA or intact, infectious provirus (8,448 nucleotides).[37] Moreover, the appearance of cortical bone DNA examined by agarose gel electrophoresis suggested that the mechanism of ETO retroviral inactivation may be via nucleic acid degradation.[12]

Abrogation of infectious retrovirus by ETO treatment of FeLV-infected bone was confirmed in the present in vitro study. Absence of FeLV p27 antigen and provirus in ETO group replicates was documented at all 4 passages. Moreover, the absence of protective host immunity in this cell culture model permitted a theoretically more robust test for detection of infectious virus.

Previously reported results of an in vivo study[12] with glycerol-treated bones from the same FeLV-infected donor cats differed from results of the present in vitro study. Transmission of retrovirus from glycerol-treated, FeLV-infected bone was documented in 1 of 5 in vivo recipients, but was not observed in any of the 5 in vitro replicates.[12] A possible explanation for this discrepancy was that the hygroscopic action of residual glycerol coating the cortical bone samples caused cell lyophilization with resultant slowing of cellular and viral replication.[38] Cells in the glycerol-treated group in the study reported here required 3 (donor cats 120 and 128), 7 (donor cat 118), or 9 (donor cats 121 and 127) additional days to reach confluence during the first passage when glycerol concentrations were highest, but thereafter became confluent at the same rate as other groups. Because FeLV replicates in rapidly dividing cells, reduction in the rate of cellular replication would cause a suboptimal environment for viral replication. Although a small amount of FeLV provirus was amplified from 1 glycerol-treated group cell sample at passage 1, virus particles may have been rendered unable to replicate in this microenvironment. In contrast, the in vivo microenvironment afforded optimal opportunities for cellular and viral replication via a rapidly dividing, diversely and densely cellular population with copious fresh blood flow.

Preservation of cortical bone allografts with a 98% solution of glycerol for 4 weeks cannot be recommended for retroviral sterilization because of detection of in vivo transmission of FeLV to an SPF cat following implantation of a glycerol-preserved bone allograft.[12] Moreover, slowed cellular replication caused by the presence of glycerol likely inhibited viral replication in vitro. The effects of different glycerol concentrations, storage temperatures, or times on retroviral inactivation are unknown.

Observed differences in retroviral transmission from glycerol-preserved bones to cell cultures versus allograft recipient animals highlight the importance of conducting both in vitro and in vivo studies. Paired in vitro and in vivo studies maximize the capacity to arrive at accurate conclusions. If the in vitro study presented here had been performed in isolation, it would have been possible to reach the erroneous conclusion that glycerol treatment provided reliable antiretroviral activity.

Results of the present in vitro study and the previously reported in vivo study[12] provided insight for human and veterinary medicine. The orthopedic community may be cautiously optimistic regarding the safety and efficacy of ETO sterilization to block retroviral transmission from infected bone allografts. Moreover, this report provides a useful starting point for investigating strategies to prevent transmission of canine diseases following allograft transplantation, a procedure that is more commonly used in dogs than cats. Rigid donor-screening practices continue to be an important aspect for preventing disease transmission from transplantation of infected allografts.

[a]ViraCHEK/FeLV antigen test kit, Synbiotics Corp, San Diego, Calif.
[b]Bio-Kinetics reader EL 312, Bio-Tek Instruments Inc, Winooski, Vt.
[c]QIAmp tissue kit (250), QIAGEN Inc, Santa Clarita, Calif.
[d]DNA miniflourometer, model TKO 100, Hoefer Scientific Instruments, San Francisco, Calif.
[e]Perkin-Elmer ABI prism 7700 sequence detector, Perkin Elmer Applied Biosystems, Branchburg, NJ.
[f]Macromolecular Structure Facility, Department of Biochemistry, Michigan State University, East Lansing, Mich.
[g]Integrated DNA Technologies, Coralville, Iowa.
[h]Epi Info, version 1.0, Centers for Disease Control, Atlanta, Ga. Available at: www.cdc.gov/epiinfo.

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