Circadian Rhythms of Granzyme B, Perforin, IFN-γ, and NK Cell Cytolytic Activity in the Spleen: Effects of Chronic Ethanol

Alvaro Arjona, Nadka Boyadjieva and Dipak K. Sarkar

*J Immunol* 2004; 172:2811-2817; doi: 10.4049/jimmunol.172.5.2811

http://www.jimmunol.org/content/172/5/2811

---

Why *The JI*?

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

References

This article **cites 55 articles**, 10 of which you can access for free at: http://www.jimmunol.org/content/172/5/2811.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852. Copyright © 2004 by The American Association of Immunologists. All rights reserved. Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Circadian Rhythms of Granzyme B, Perforin, IFN-γ, and NK Cell Cytolytic Activity in the Spleen: Effects of Chronic Ethanol

Alvaro Arjona, Nadka Boyadjieva, and Dipak K. Sarkar

Recent studies show that alterations in the body’s biological rhythms can lead to serious pathologies, including cancer. Acute and chronic ethanol consumption impairs the immune system by causing specific defects in the cellular components of the innate immune response and by creating increased risk and susceptibility to infections and cancer. NK cells are critical for immune surveillance against infected and malignant cells. To assess whether NK cell function follows a circadian trend and to determine ethanol effects on this rhythm, we measured, over a 24-h period, mRNA and protein levels of granzyme B, perforin, and the cytokine IFN-γ, as well as NK cell activity, in the splenocytes of ad libitum-fed, pair-fed, and ethanol-fed Sprague Dawley male rats. Circadian rhythms were found in mRNA and protein levels of granzyme B, perforin, and IFN-γ. A circadian pattern was also detected in NK cell cytolytic activity. Our data further demonstrated how chronic ethanol suppressed NK cell activity by directly disrupting the circadian rhythms of granzyme B, perforin, and IFN-γ. These findings identify the circadian functions of splenic NK cells and show the vulnerability of these rhythms to chronic ethanol. The Journal of Immunology, 2004, 172: 2811–2817.

Circadian rhythms describe biological phenomena that oscillate within a 24-h cycle. These rhythms provide a temporal frame necessary for adequate homeostasis. By anticipating both environmental and internal changes, cells (and organisms) can efficiently program their physiological tasks. It is believed that peripheral cells are able to adjust their physiology to a circadian rhythm (1). Cellular rhythms are originated by genetic elements (clock genes) organized in autoregulatory transcription-translation feedback loops that form the cellular core oscillator. This oscillating machinery may control the expression of the so-called clock-controlled genes, and, ultimately, generate the circadian rhythms in physiology and behavior (2).

In mammals, the suprachiasmatic nucleus (SCN) is considered the central pacemaker controlling the circadian rhythms in the body (3). This hypothalamic master clock is entrained by external cues called zeitgebers. The main external zeitgeber is the photic input, coming from the eyes to the SCN through the retinohypothalamic tract, carrying information from the light/dark cycle. However, SCN entrainment can be complemented by internal signals such as melatonin. In higher organisms, external cues are unable to reach the peripheral cellular oscillators, so the SCN has to program and coordinate the peripheral clocks through neural and endocrine pathways.

Alterations in circadian rhythms can lead to serious pathologies such as sleep disorders, cardiovascular disease, and depression. In addition, recent studies show a connection between altered circadian rhythms and cancer. Even though previous research pointed to a direct influence of diurnal changes on the immune system, the underlying mechanisms governing immune circadian rhythmicity and its functional implications are still largely unknown.

NK cells are critical for immune surveillance against fungal, bacterial, and viral infections. They also play a vital role in cellular resistance to malignancy and tumor metastasis. NK cells can destroy their target cells by calcium-dependent release of cytolytic granules, by activation of the Fas (CD95) pathway, or through contact with TNF-α. Among these mechanisms, the release of cytolytic granules containing granzymes (particularly granzyme B) and perforin is the major mechanism used for killing the target cell (14, 15). Perforin creates transmembrane pores in the target cell membrane thereby allowing the entry of granzyme B, which then activates the apoptotic pathways of the caspases. NK cells differ from the other cytotoxic effector cell types (CTL) in two major ways—they kill the target cells in a non-MHC-restricted fashion without the need for previous in vitro or in vivo activation, and only NK cells constitutively express the lytic machinery.

The immunosuppressive effect of chronic ethanol is well documented. There is a strong association between ethanol consumption and both increased cancer risk and infection morbidity. We have recently shown how chronic ethanol reduces granzyme B, perforin, and NK cell cytolytic activity, however, there is no report showing a direct influence of ethanol on the circadian rhythm of NK cells. Our aim was to test whether NK cell function follows a circadian rhythm and, if so, to analyze the effect of chronic ethanol consumption. For that purpose, over a 24-h period, we measured mRNA and protein levels of granzyme B, perforin, and IFN-γ, as well as NK cell cytolytic activity in the splenocytes of ad libitum-fed, pair-fed, and ethanol-fed Sprague Dawley male rats.

Materials and Methods

Animals

Sprague Dawley male rats, 2–3 mo of age, were maintained on a 12-h light/dark cycle (lighting period from 7:00 a.m. to 7:00 p.m.) and were either ad libitum-fed rodent chow, fed an ethanol-containing liquid diet (8.7% v/v), or pair-fed an isocaloric liquid diet (Bio-Serv, Frenchtown, New Jersey). Animals were divided into three groups: ad libitum-fed, pair-fed, and ethanol-fed. The ethanol-fed group received an ethanol-containing liquid diet containing 8.7% v/v alcohol. The pair-fed group received an isocaloric liquid diet without alcohol. All animals were housed in individual cages in a facility maintained at 22°C with a 12-h light/dark cycle. Food and water were provided ad libitum in the case of ad libitum-fed animals and pair-fed animals, whereas the ethanol-fed group was pair-fed. The experiment was conducted in accordance with the institutional guidelines for animal care and use. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Institutes of Health Grants AA00220 and AA12642.

2 Address correspondence and reprint requests to Dr. Dipak K. Sarkar, Endocrinology Program, Rutgers, State University of New Jersey, New Brunswick, NJ. E-mail address: sarkar@aesop.rutgers.edu

3 Abbreviations used in this paper: SCN, suprachiasmatic nucleus; ZT, zeitgeber time.
NJ). Graduated ball-barrel cylinders containing the freshly prepared diet were placed in the animal cages 1 h before the dark period. After 2 wk of treatment, five to six rats per time point were euthanized at 6:00 a.m., 10:00 a.m., 2:00 p.m., 6:00 p.m., 10:00 p.m., and 2:00 a.m. These time points correspond with zeitgeber times (ZT) 3, 7, 11, 15, 19, and 23, respectively. Spleens were immediately collected and aliquoted for further processing. Throughout the study, animal care and treatment protocol complied with National Institutes of Health policy, were in accordance with institutional guidelines, and were approved by the Rutgers Animal Care and Facilities Committee.

**Measurement of mRNA levels of granzyme B, perforin, and IFN-γ**

RNA from spleen tissue aliquots was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). One microgram of total RNA was reverse transcribed to the first strand cDNA using Superscript II First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Relative quantification of mRNA levels of the cytolytic factors granzyme B, perforin, and the cytokine IFN-γ was performed by real-time PCR (SYBR Green Assay; Applied Biosystems, Foster City, CA) using an ABI prism 7700 Sequence Detector. The following primer sequences (5′-3′) were used (22): granzyme B sense, GCCAGCGAGCCCCTGCTCATCA; IFN-γ sense, ACATCCGG; perforin sense, GCATCGGTGCCCAAGCCAGTG, anti-sense, GTG. Analyses were done using the standard curve method with GAPDH as the normalizing endogenous control. Relative mRNA levels at each time point were calculated as a percentage of the maximum value observed over the 24-h period.

**Measurement of protein levels of granzyme B, perforin, and IFN-γ**

Spleen tissue aliquots were lysed in lysis buffer containing 150 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM MgCl2, 1% Triton X-100, 10% glycerol, and protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO). After protein quantification (Bio-Rad, Hercules, CA), whole spleen lysates were subjected to standard SDS-PAGE electrophoresis, and proteins were transferred to polyvinylidene difluoride membranes following usual procedures. Blots were probed with granzyme B mAb (BD Biosciences, Franklin Lakes, NJ), perforin polyclonal Ab (Torrey Pines Biolabs, San Marcos, CA), and IFN-γ polyclonal Ab (Chemicon International, Temecula, CA), and actin mAb (Oncogene, San Diego, CA) for normalizing purposes. After chemiluminescence detection (ECL plus; Amersham, Arlington Heights, IL), densitometric analyses were performed using Scion (Frederick, MD) Image software. Relative protein levels were calculated as a percentage of the maximum value observed in each blot.

**Measurement of splenic NK cell cytolytic activity**

To assess NK activity, standard 4-h 51Cr release assays were performed as described previously (20). Briefly, splenocytes were isolated from fresh spleen tissues and then challenged to 51Cr-labeled YAC-1 lymphoma cells. Percentages of activity at four E:T ratios (200:1, 100:1, 50:1, and 25:1) were converted to lytic units according to Pross et al. (23). Assays were done in quadruplicate.

**Statistical analyses**

One-way ANOVA with the Dunnett post-test was used to assess differences between time points of the same group. In the Dunnett post-test, the lowest value was chosen as the control. To assess differences between pair-fed and ethanol-fed groups, two-way ANOVA with Bonferroni post-test was performed. A p value < 0.05 was considered statistically significant.

**Results**

**Circadian rhythm in mRNA levels of granzyme B, perforin, and IFN-γ**

Splenocytes from ad libitum-fed rats showed a robust circadian rhythm in mRNA levels of granzyme B and perforin (Fig. 1A and B). Both cytolytic factors presented the highest levels at ZT3 (Dunnett test, p < 0.05), and the lowest levels at ZT15. Similarly, IFN-γ mRNA levels in the splenocytes of ad libitum rats peaked significantly at ZT3 (Dunnett test, p < 0.05) and presented the lowest levels at ZT15 (Fig. 1C). However, the circadian pattern was not as well defined for granzyme B and perforin as for IFN-γ.

**Effect of chronic ethanol on the mRNA circadian rhythms of granzyme B, perforin, and IFN-γ**

Splenocytes from pair-fed rats showed a peak in mRNA content of granzyme B, perforin, and IFN-γ at ZT7 (Dunnett test, p < 0.05; Fig. 2). This is 4 h later than the peak observed in ad libitum-fed rats. Nevertheless, the mRNA circadian rhythms of granzyme B,
perforin, and IFN-γ in the splenocytes of pair-fed rats were consistent with the rhythms observed in ad libitum-fed rats, with the highest levels during the light phase and lowest levels during the dark phase. Chronic ethanol blunted the mRNA circadian rhythms of granzyme B, perforin, and IFN-γ; no significant peaks were observed in their mRNA content (Dunnett test, \( p < 0.05 \); Fig. 2).

**Circadian rhythm in protein levels of granzyme B, perforin, and IFN-γ**

Splenocytes from ad libitum-fed rats showed a circadian rhythm in protein levels of granzyme B, perforin, and IFN-γ (Figs. 3 and 4). Cytolytic factors granzyme B and perforin peaked significantly at ZT15 (Dunnett test, \( p < 0.05 \); Fig. 4, A and B). Interestingly, the peaks in the protein content of these two cytolytic factors coincided with their lowest levels of mRNA (Fig. 1, A and B). The peak in IFN-γ protein content was found at ZT19 (Dunnett test, \( p < 0.05 \)), and the levels during the dark phase time points remained significantly higher than the levels during the light phase time points (Dunnett test, \( p < 0.05 \)). In summary, the splenic protein content of granzyme B, perforin, and IFN-γ in ad libitum-fed rats all showed a circadian rhythm with the highest levels during the dark phase and the lowest levels during the light phase.

**Effect of chronic ethanol on the protein circadian rhythm of granzyme B, perforin, and IFN-γ**

As seen in ad libitum rats, splenocytes from pair-fed rats showed a peak in the protein content of granzyme B and perforin at ZT15 (Dunnett test, \( p < 0.05 \); Figs. 3 and 5, A and B). IFN-γ protein levels peaked at ZT23 (Dunnett test, \( p < 0.05 \); Fig. 5C), 4 h later than the peak observed in ad libitum-fed rats. Nevertheless, the circadian rhythm of IFN-γ protein content fairly matched the one found in ad libitum rats, with the lowest levels at the end of the light phase and higher levels during the dark phase. Chronic ethanol blunted the circadian rhythm in the protein content of granzyme B, perforin, and IFN-γ; the amplitudes were drastically reduced and no significant peaks were found in the ethanol-fed group (Dunnett test; Fig. 5). Furthermore, the protein levels of granzyme B, perforin, and IFN-γ, detected in the splenocytes of ethanol-fed rats were significantly lower than the levels in pair-fed rats at ZT7, the time of highest mRNA content (Bonferroni test, \( p < 0.05 \); Fig. 2, A and B).
rats, were significantly lower than the levels in pair-fed rats at the time when the highest protein levels were detected (Bonferroni test, $p < 0.05$; Fig. 5). Interestingly, IFN-\(\gamma\) protein content in the splenocytes of ethanol-fed rats was significantly higher than in the pair-fed animals at ZT7 and ZT11 (Bonferroni test, $p < 0.05$; Fig. 5C).

**Circadian rhythm of NK cell cytolytic activity**

Splenic NK cells from ad libitum-fed rats showed a circadian rhythm in cytolytic activity with a significant peak at ZT19 (Dunnett test, $p < 0.05$; Fig. 6A), coinciding with the middle of the dark period. The lowest levels of NK activity were found at ZT11 and ZT15. The cytolytic activity levels at the rest of the time points were also significantly elevated.
Splenic NK cell cytolytic activity as measured by a standard 51 Cr rhythm in rat splenocytes. This diurnal pattern was also detected in levels of granzyme B, perforin, and IFN-γ. The data presented in this study show, for the first time, how the levels of granzyme B, perforin, and IFN-γ all follow a circadian rhythm in rat splenocytes. This diurnal pattern was also detected in splenic NK cell cytolytic activity as measured by a standard 51Cr assay. In a nonstimulated splenocyte population, only NK cells constitutively express the lytic machinery (24–26). Therefore, granzyme B and perforin mRNA and protein levels, detected in the spleen, can be attributed exclusively to NK cells.

Perforin and granzymes have long been recognized as fundamental components of the granule-mediated cytotoxic activity (15). By adjusting the levels of granzyme B and perforin in a circadian fashion, splenic NK cells provide themselves with the logistics required to follow their physiological rhythm in cytolytic activity. In fact, the maximum protein levels of cytolytic factors were found 4 h before the peak in NK activity, suggesting an anticipation mechanism.

Although NK cells are not the sole source of IFN-γ (27), the mRNA and protein levels of this cytokine matched the circadian patterns found for granzyme B and perforin. IFN-γ protein content was significantly higher during the dark period, in accordance with maximum levels of NK cytolytic activity. These data suggest a coordination of the synthesis and accumulation of IFN-γ with the rhythms of granzyme B, perforin, and NK cytolytic activity. Even though we measured the protein content within the splenocytes, not the actual release, this is an expected finding because IFN-γ is a potent enhancer of NK cell function (28).

In accordance with our own work, previous studies with rat spleens point to increased NK activity during the dark period (29–31). Using Fischer rats, Shakhar et al. (32) showed an increase in tumor clearance effectiveness during the dark phase. They also concluded that during the dark period, the percentage of NKR-P1bright cells within the splenocytes increase 28.9% when measured by FACS. Using this same procedure, we did not find a significant difference in the percentage of NKR-P1bright cells within the spleens of ad libitum-fed rats (7.8 ± 0.7 and 7.1 ± 0.5, respectively). McNulty et al. (30) did not find significant changes of NK cell numbers during the day either. Our previous work demonstrated that 2-wk alcohol treatment does not produce a significant change in the total splenocyte number, percentage of NK cells, or in NK cell numbers in the spleen (20). Other authors have also concluded that ethanol minimally affects the anatomical distribution of NK cells (33). In addition, recently published work from our laboratory shows that 2-wk alcohol treatment does not have any significant effect on the percentage or the number of CD3+CD45R0− cells within the spleen when compared with ad libitum-fed and pair-fed animals (34). Thus, we think that the circadian rhythms detected in mRNA and protein levels of granzyme B, perforin, IFN-γ and NK activity, as well as the effects of ethanol on these rhythms, seem to be due mainly to inner cellular changes rather than to variations in the distribution of NK cells or other lymphocytes within the spleen.

Most of the studies in humans demonstrate that, as in rats, NK activity peaks during the period of wakefulness (35–37). During this period, organisms face daily challenges, stressors, exposure to pathogens, and the like. It is not surprising then that the immune system, to optimize its function when it is most needed, could have developed circadian regulation.

Granzyyme B, perforin, and IFN-γ mRNA levels peaked at the beginning of the light phase (ZT3) in ad libitum-fed rats. In the pair-fed group, the peak was also found during the light phase, but 4 h later (ZT7). The daily feeding process may act as a resetting event for the central clock, thereby producing a shift in the mRNA peak. Also, the calorie restriction associated with the liquid diets can affect the immune system (9). However, in both ad libitum and pair-fed rats, mRNA levels were consistently higher at the beginning of the light period (ZT3–ZT7); were lower at the beginning of the dark phase (ZT19), and, started to rise again at the end of the dark period (ZT23).

Also, circadian profiles of the protein levels of granzyme B, perforin, and IFN-γ matched. Their lower levels were all found in the light period and their higher levels during the dark phase, preceding the peak in cytolytic activity. Interestingly, the peak in granzyme B and perforin protein levels, consistently found in ad

![Graph A](http://example.com/graphA.png)

**FIGURE 6.** Circadian rhythm in cytolytic activity of splenic NK cells of ad libitum-fed (A), pair-fed, and ethanol-fed rats (B). Splenocytes were freshly isolated from spleen aliquots and challenged with 51Cr-labeled YAC-1 lymphoma cells at four E:T ratios from 25:1 to 200:1. Cytolytic activity was determined in lytic units. Data are mean ± SEM of four to six animals per time point and group. *, p < 0.05 significantly different from the lowest value in the same group as per one-way ANOVA with Dunnett post-test. #, p < 0.05 significant difference between groups as per two-way ANOVA with Bonferroni post-test. Samples were collected at the following clock times: 6:00 a.m., 10:00 a.m., 2:00 p.m., 6:00 p.m., 10:00 p.m., and 2:00 a.m. These time points correspond with ZT 3, 7, 11, 15, 19, and 23, respectively.

**Effect of chronic ethanol on NK cell cytolytic activity circadian rhythm**

As in the ad libitum-fed group, both pair-fed and ethanol-fed groups showed a circadian rhythm in NK cytolytic activity with a significant peak at ZT19 (Dunnett test, p < 0.05; Fig. 6B). Likewise, the lowest activity levels in all three groups were found at the end of the light period/beginning of the dark phase (ZT11–ZT15). Chronic ethanol treatment significantly reduced the NK activity levels in five of six time points (Bonferroni test, p < 0.05; Fig. 6B). However, splenic NK cells from ethanol-fed rats were able to maintain the circadian rhythm in cytolytic activity, with a significant peak at ZT19 (Dunnett test, p < 0.05; Fig. 6B).

**Discussion**

The data presented in this study show, for the first time, how the levels of granzyme B, perforin, and IFN-γ all follow a circadian rhythm in rat splenocytes. This diurnal pattern was also detected in splenic NK cell cytolytic activity as measured by a standard 51Cr assay. In a nonstimulated splenocyte population, only NK cells constitutively express the lytic machinery (24–26). Therefore, granzyme B and perforin mRNA and protein levels, detected in the spleen, can be attributed exclusively to NK cells.

Perforin and granzymes have long been recognized as fundamental components of the granule-mediated cytotoxic activity

![Graph B](http://example.com/graphB.png)
libitum and pair-fed rats at ZT15, coincided with their lowest levels of mRNA. Thus, it is possible that the transcription and translation cycles for these two cytolytic factors are tightly autoregulated by a negative feedback loop adjusted to a 24-h rhythm. It is also possible that a direct intervention of the cellular clock machinery occurs. For this reason, we are currently conducting experiments to assess the expression of clock genes in the spleen.

It is yet to be determined whether the circadian rhythms found in cytolytic factors, IFN-γ, and NK cytolytic activity are endogenously generated, or if they are, to some extent, centrally governed. NK cell circadian rhythm in the spleen might be entrained by the central clock—the SCN—through hormonal and/or neural cues. Glucocorticoids, prolactin, melatonin, pro-opiomelanocortin-derived peptides and catecholamines are all substances that are released in a circadian fashion and have been proven to affect NK cell function (20, 38–41). We have previously shown the strong influence of the opioid β-endorphin on NK cytolytic activity (20, 34). We have found recently that pro-opiomelanocortin transcript levels in the hypothalamic arcuate nucleus follow a circadian pattern that correlates with the circadian rhythm in NK activity (42).

In humans, it has been shown that plasma levels of β-endorphin and other endogenous opioid peptides, like α-melanocyte-stimulating hormone, follow a circadian profile (43). In addition, circadian variations in cortisol plasma levels have been strongly associated with circadian variations of the immune system especially NK function (44). However, Depres-Brunner et al. (45), showed persistent rhythms in T lymphocyte blood counts in rats deprived of circadian clock outputs such as catecholamines. Further research is needed to identify the role of neuroendocrine hormones in mediating the influence of the central clock on NK rhythm.

Chronic alcohol consumption has been largely associated with alterations in the immune system (46, 47), increased susceptibility to opportunistic microbes (48), and higher incidence of certain forms of cancer (49). In addition, a large number of studies indicate that ethanol reduces NK cell cytotoxicity in animals and humans (20, 50–54). The findings reported in this work describe how ethanol can disrupt the physiological rhythms of cytotoxic factors and NK activity, and this can be an important mechanism underlying the higher incidence of infections and cancer in alcoholic patients.

The present data provide novel evidence of the ability of ethanol to disrupt the physiological circadian rhythms of granzyme B, perforin, and IFN-γ, specifically by hampering the occurrence of the peaks in their mRNA and protein levels. Because our previous work demonstrated that ethanol treatment for 2 wk does not affect the splenocyte number, total NK cell number, or percentage of NK cells within the splenocytes (20), this represents a specific alteration of NK cell circadian regulation.

NK cells with an altered rhythm in granzyme B and perforin are not able to anticipate the accumulation of critical cytotoxic components. Thus, the killing capacity of these NK cells is seriously compromised. Our findings demonstrate that even though splenic NK cells from alcoholic rats are able to maintain a circadian pattern, their cytolytic activity is significantly diminished. Hence, disrupted rhythms of granzyme B, perforin, and IFN-γ in alcoholic individuals may underlie the higher incidence of infections and cancer within this population. Likewise, disrupted rhythms in the synthesis and accumulation of cytotoxic factors and IFN-γ may be the cause of the higher incidence of cancer that has been discovered lately in people, such as night-shift workers and flight crews, with altered daily rhythms (6).

In summary, we report, for the first time, that mRNA and protein levels of the cytolytic factors granzyme B and perforin, as well as the cytokine IFN-γ, follow a physiological circadian rhythm that concurrently drives the circadian rhythm in NK cell cytolytic activity. Furthermore, we have demonstrated how chronic ethanol consumption is able to suppress NK cell activity by directly disrupting the circadian rhythm of granzyme B, perforin, and IFN-γ. In the last few years, the medical practice consisting of timed administration of drugs based on biological rhythms has experienced extensive development, especially in cancer therapy (55, 56). The information on the mechanisms orchestrating the circadian rhythm of NK cell function, obtained from this study and from further investigations, may be helpful in the prevention and treatment of immune-related diseases.

Acknowledgments

We thank Michael Poplawski and Jason Marano for their help with the animal work and sample collection.

References


