Journal of the American Association for Laboratory Animal Science
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Vol 45, No 2
March 2006
Pages 26–30

Chronic Care and Monitoring of Woodchucks (*Marmota monax*) during Repeated Magnetic Resonance Imaging of the Liver

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We monitored the development of hepatocellular carcinoma due to chronic infection with woodchuck hepatitis virus by using monthly serum samples, physical examination, and magnetic resonance imaging. The same woodchucks can be imaged repeatedly over a 1-y period by allowing the animals to recover after each experiment, thus reducing the number of animals required without compromising the quality of the data obtained. Age- and sex-matched uninfected control (n = 5) and chronically infected (n = 5) woodchucks were group-housed according to sex and infection status. Woodchucks were anaesthetized using an inhalation anesthetic (isoflurane) without premedication. During imaging, we regularly monitored heart rate, body temperature, and respiration. Tumor growth was observed using MRI, whereas the extent of hepatocyte injury was followed using serum liver enzymes. Elevated serum gamma glutamyltransferase and aspartate aminotransferase levels indicated hepatocyte injury due to tumor growth. On magnetic resonance images, the liver should appear as a well-defined, homogenous organ with defined regions of hyperintensities from larger blood vessels. Within tumor nodules, the liver appeared irregularly shaped, having heterogeneous intensity from unregulated cellular proliferation. Changes in tumor size can be monitored by imaging infected woodchucks on a regular basis. Using the imaging techniques we describe, the development of hepatocellular carcinoma can be visualized using magnetic resonance imaging, correlated to serum tests, and compared with the results from uninfected control woodchucks, thereby improving the understanding of the disease progress.

Abbreviations: AST, aspartate aminotransferase; FLASH, fast low-angle shot; HBV, hepatitis B virus; GGT, gamma glutamyltransferase; MRI, magnetic resonance imaging; WHsAg, woodchuck hepatitis virus surface antigen; WHV, woodchuck hepatitis virus

The discovery that woodchucks (*Marmota monax*) develop hepatocellular carcinoma due to chronic infection with woodchuck hepatitis virus (WHV) has been important in the field of viral hepatitis research. Woodchuck hepatitis virus has the greatest degree of genetic similarity to human hepatitis B virus (HBV) than any other member of virus family Hepadnaviridae.[6] The long-term effect on the livers of chronically infected woodchucks is also similar to that of hepatitis B virus on human livers. With the decline in research involving chimpanzees, the woodchuck model of chronic viral hepatitis will improve the understanding of viral infection and carcinogenesis and the development of novel therapeutics.[2,6,12,14] Although this species is rarely used in the lab, the woodchuck is an important animal model for the study of HBV infections in humans because tumor formation in woodchucks occurs in less than 3 y, making them a suitable model to study carcinogenesis.

Previously published papers using magnetic resonance imaging (MRI) of the woodchuck model of hepatocellular carcinoma address acute studies where the woodchucks were euthanized upon completion of the imaging.[9-11] The housing, monitoring, and imaging techniques we describe here were developed specifically for chronic experiments with woodchucks that allow for regular MRI of the same animals over a 1-y period. Chronic experimentation on woodchucks allows for better understanding of the disease process in individual animals over time, thus reducing the total number of animals required in a long-term project.

Received: 5 July 2005. Revision requested: 7 Oct 2005. Accepted: 7 Oct 2005.
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Materials and Methods

All experiments were conducted with the approval of the National Research Council Institute for Biodiagnostics's Animal Care Committee, following the guidelines of the Canadian Council of Animal Care (CCAC).

The Eastern woodchuck (*Marmota monax*) is a large, burrowing animal, which can reach an average length of 41 to 65 cm with a girth of 28 to 40 cm. Depending on the season, body weight can range from 1.4 to 2.5 kg during winter to 2.2 to 4.2 kg during summer (Figure 1).[2,15] We purchased five 1-y-old uninfected control woodchucks from the department of Molecular Virology and Hepatology Research (Faculty of Medicine, Memorial University, St John's, Newfoundland, Canada) and five 1-y-old woodchucks chronically infected with WHV from NorthEastern Wildlife (South Plymouth, NY). Woodchucks were infected with WHV within 7 d after birth to ensure the development of chronic hepatitis. As with human hepatitis B, the earlier that an individual is exposed to the virus, the greater the likelihood that it will develop a chronic infection leading to terminal liver cancer (hepatocellular carcinoma).[6] Woodchucks were purchased at 1 y postinfection, with serum levels of WHV surface antigen (WHsAg) that indicated chronic infection.

Primary housing. Although in their natural habitat woodchucks are primarily asocial territorial animals, in captivity they can be housed individually, in breeding pairs, or by gender. To

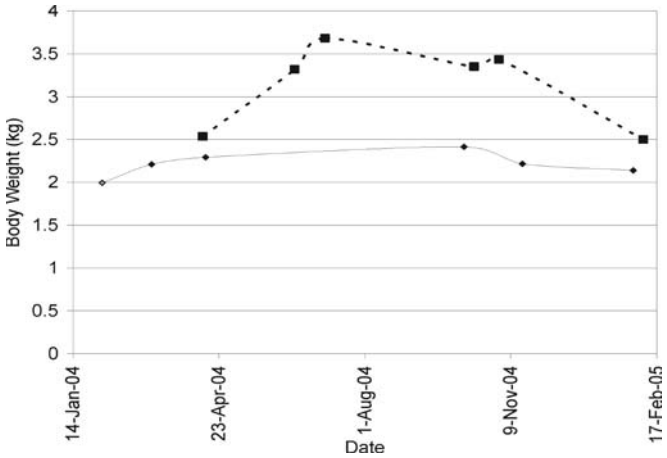

Figure 1. Changes in average body weight of woodchucks (control, solid line, n = 5; infected, dashed line, n = 5) over 1 y. In the wild, woodchucks hibernate through winter. Even in control laboratory conditions, woodchucks undergo a period of reduced food and water intake each winter, causing weight loss that is not a result of chronic infection with woodchuck hepatitis virus.

avoid aggressive behavior, breeding, and the spread of WHV, the woodchucks were separated among 4 CCAC-approved rooms (each 7.2 m^2 [3.7 m × 2.0 m]) according to gender and infection status; control males, control females, infected males, and infected females were housed in separate rooms. The cement floor was covered with aspen shavings and shredded recycled paper, which was used by the woodchucks to hide bodily wastes, typically in a corner or behind nest boxes.[2] Shavings were added as necessary; shredded paper was replaced every 3 to 4 d.

Large polyvinylchloride tubing (45 cm × 45 cm × 15 cm) and stainless steel nesting boxes (50 cm × 53 cm × 20 cm) were provided in each room as environmental enrichment, allowing a burrow-like environment for the woodchucks. As woodchucks' incisors grow continuously, chemically untreated wooden benches constructed with wooden pegs were included for the woodchucks to gnaw on to avoid impaction of the teeth.

Room temperature was maintained at 20 to 22 °C. Humidity was maintained between 45% to 55% to prevent the drying and flaking of the woodchucks' skin, as well as cracking of the pads of the feet, which can become a source of infection.[2] Lighting in the rooms was set on 12-h timers with an incandescent bulb activated 30 min before the fluorescent lights turned on and for 30 min after the fluorescent lights were turned off, to simulate sunrise and sunset.

Food. Woodchucks were provided with filtered water and a mixture of rabbit and woodchuck pellets (Prolab Hi-Fiber Rabbit 5P25, Harlan Teklad Woodchuck Diet #7778, Madison, WI) ad libitum. Food and water were provided in multiple stainless steel food dishes to prevent aggressive competition between animals in the room. The food dishes had wide flat bases, which prevented the woodchucks from tipping the bowls over.[2,15]

Animal identification. In group housing, individual animals were identified using unique shave patterns in the fur. As woodchucks only grow fur twice a year, patterns were reshaved in the spring and fall. Tattoos and ear tags were not used because tattoos were not visible unless individual animals were caught and picked up.[15] Ear tags were removed because they potentially contained ferromagnetic metals that would interfere with the magnetic field of the MRI machine or could be ripped out during a fight.

Anesthesia. Prior to imaging, each woodchuck was captured in an induction chamber, a heavy-gauge plastic box (48 cm × 21 cm × 20 cm) with a locking lid. Isoflurane (Aerrane, Pharmaceutical Partners of Canada Richmond Hill, Ontario, Canada) was introduced into the induction chamber though tubing connected to the side of the box via an adaptor and pre-drilled holes. Exhaust was vented through a separate exhaust line on the other side of the container and connected to a passive vacuum. Deep-plane anesthesia was achieved within 5 min (5% isoflurane, 5 l/min 100% O_2). The anesthetized woodchuck was removed from the induction chamber and positioned on its dorsal surface for physical examination and blood draws. Anesthesia was maintained (3.5% isoflurane, 2 l/min O_2) during the physical examination by using a full facemask with a rubber diaphragm (inner diameter, 52 mm).

Preimaging physical examinations. The abdomen of the anesthetized woodchuck was palpated to detect any firmness or tumors. The nails were clipped, and the feet were moisturized with Hibitane cream (1% chlorhexidine, Wyeth Ayerst Veterinary Laboratories, Zeneca Pharma, Guelph, Ontario, Canada) preventing the pads drying due to the aspen shavings and cement floor. Eye lubricant was applied to prevent eye dryness during imaging.

Blood was drawn prior to each imaging session by venipuncture of the tarsal vein of a hindfoot by using a 21 1/2-gauge, 1-in. needle.[2,4,7] The foot and inner leg was shaved, cleaned with Hibitane Scrub (Zeneca Pharma, Guelph, Ontario, Canada) and rinsed with isopropyl alcohol. We collected 2 to 5 ml blood into phlebotomy tubes and centrifuged them to separate the serum from the red blood cells. Pressure was applied to the puncture site to prevent the formation of a hematoma. The serum samples were tested for viral titer, WHsAg, gamma glutamyltransferase (GGT), and aspartate aminotransferase (AST).

Serum samples were tested for viral load using reverse transcription–polymerase chain reaction amplification (RT-PCR; Applied Biosystems 7500 Fast RT-PCR System), and the serum hepatic enzymes GGT and AST were measured using an automatic analyzer (Vitros 250, Ortho-Clinical Diagnostics, Mississauga, Ontario, Canada). RT-PCR is a method to quantitate the amount of viral DNA present in the serum obtained from the woodchucks. A oligonucleotide complementary to a section of WHV contains a 5′ fluorescent reporter dye and a 3′ quencher. During DNA amplification, the oligonucleotide is degraded by DNA polymerase, allowing the fluorescent reporter dye to emit light proportional to the amount of dye released which, in turn, is proportional to the amount of DNA produced. By using a standard curve created from known DNA concentrations, the amount of viral DNA present in the serum can be calculated.

WHsAg was quantitated using sandwich enzyme-linked immunosorbent assays. For this test, a solid substrate is coated in anti-WHsAg antibodies; serum from infected animals containing WHsAg is applied, followed by a secondary anti-WHsAg antibody conjugated with alkaline phosphatase. The absorbance of the alkaline phosphatase at 450 nm is proportional to the concentration of WHsAg in the serum tested. Results are reported as a ratio of the absorbance of infected serum to uninfected serum, where uninfected serum does not contain WHsAg acts as a standard.

MRI. The anesthetized woodchuck was positioned on its ventral surface in a removable cradle. The cradle was inserted in the center of a custom-made doubly tunable quadrature volume coil for proton imaging and phosphorus spectroscopy.[13] Anesthesia was maintained at 2% to 3% isoflurane with a flowrate of 2 l/min O_2 via a flexible small-animal full facemask (Harvard Apparatus Holliston, ME) and held in place with stockinette

tubing (3M Health Care, St Paul, MI). Exhaust was vented via an active scavenging vacuum through an outlet on the side of the mask. Respiration and heart rate were monitored on a Grass recorder (Grass Instrument, Quincy, MA) connected to a receiver coil positioned on the back of the animal. The coil detects minor fluctuations in the magnetic field due to motion from each breath. Outside the magnet, a stethoscope connected to extension tubing was attached to an esophageal tube positioned beneath the animal's chest. The esophageal tube was used to confirm the heart rate and respiration detected by the Grass recorder.[5] The woodchuck's temperature was measured rectally with a thermocouple and maintained between 36.5 to 38.0 °C by using a circulating water bath connected to Tygon (Cole Parmer Instrument, Chicago, IL) tubing that lined the bottom of the cradle in a series of loops. The animal's body temperature was maintained by adjusting the temperature of the water bath. Anesthesia was maintained at 2.5% and adjusted depending on respiratory rate and heart rate. The total anesthesia time was 2.5 h.

All MRI experiments were performed in a 7T horizontal bore magnet (Magnex, Yarnton Oxfordshire, UK) equipped with an Avance DRX Bruker console (Rheinstetten, Germany). A sagittal fast low-angle shot (FLASH) scout proton image was acquired to determine the location of the liver. From this sagittal image, a 2-cm axial slab was chosen that incorporated the largest portion of liver tissue without signal contamination from surrounding organs. An axial FLASH image was acquired in the middle of the slab to be used as the pilot image for 2-dimensional chemical shift imaging.[5]

Upon completion of imaging, the animals were allowed to recover from anesthesia by returning them to the induction chamber and manually stimulated by rubbing their bodies. The lid of the induction chamber was removed, allowing the animal to breathe room air. Two drops of eye lubrication were administered, and 20 cc saline was injected subcutaneously to prevent dehydration. Once the woodchuck's righting reflex had recovered sufficiently and accepted a food reward (arrowroot cookie), it then was returned to group housing within an hour of being removed from anesthesia.

Animals chronically infected with WHV were euthanized with intravenous injection of Euthansol (Schering-Plough, Point Claire, Quebec, Canada) when a tumor could be palpated, the animal displayed abdominal guarding, and serum GGT levels exceeded 200 U/l.

Results represent the mean value from infected and control animals, and were considered significant with a P value less than 0.05 using a Student's t test. (Statistica, Tulsa, OK).

Results

During the preimaging examination, the abdomen was palpated to detect any tumor growth. Early changes of the liver of infected animals could be palpated as a nonspecific firmness of the abdomen. Histologic examination of tumors removed during necropsy showed fibrous encapsulation of the tumor from the surrounding parenchyma.

The rate of isoflurane consumption depended on the season, with higher levels required during the summer months when respiration rates were higher. The average respiration rate during winter was 3 to 5 breaths per min; whereas during summer it was 8 to 10 breaths per min. The heart rate was maintained at 180 to 210 beats per min. Because of the animals' fluctuating respiration rates throughout the year, the levels of isoflurane were closely monitored and adjusted throughout the MRI to maintain a regular respiration rate. Unlike that of other animal species, the respiration of anesthetized woodchucks was irregular: during winter months, often 2 breaths occurred in quick succession, followed by a period as long as 20 s without breathing. During summer, when respiration rates are higher, breathing was more regular and predictable.

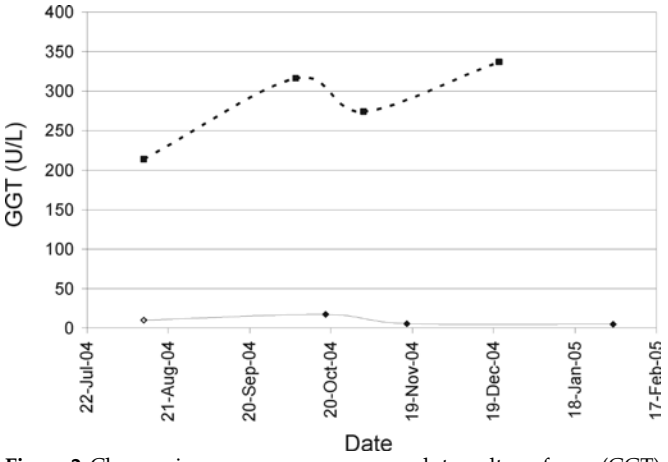

Figure 2. Changes in average serum gamma glutamyltransferase (GGT) over 1 y in control woodchucks (solid line, n = 5) and those infected with woodchuck hepatitis virus (dashed line, n = 5).

In clinical situations, human patients are instructed to breathhold during imaging, reducing motion artifacts due to breathing. Because the woodchucks were not intubated, breathing was irregular, and gradient echo images were acquired because they are a faster imaging sequence than conventional T1 or T2 images. Although image quality is poorer than with T1 or T2 images, the tumor could still be identified from surrounding normal tissue as the region of heterogeneity within the liver.

Despite the avoidance of hibernation, a woodchuck's weight will fluctuate throughout the year.[2] In our facility, we find that woodchucks consume food on a yearly cycle; this pattern occurred under ad libitum feeding, constant temperatures, and unvarying ratios of light and dark. Increases in activity, body temperature, vocalizations, food intake, and weight were seen during the months of April through October and decreases during the months of November through March. The weight gain (mean ± standard error) (Statistica software, Tulsa, OK) from April to October of uninfected woodchucks was 9.96% ± 3.50% whereas the average weight loss during November to March was –10.27% ± 1.59%. A similar pattern was seen in the infected woodchucks (Figure 1).

Uninfected control woodchucks had an average viral load of 1005.3 copies/μl, whereas infected woodchucks had an average viral load of 8.29×10^6 copies/μl ($P < 0.004$). The trace amounts of viral load in the serum of control woodchucks represent the nonspecific background noise of the RT-PCR.

Infected woodchucks had an average WHsAg level of 6.72 ± 0.837 absorbance units as calculated by ELISA (Bio-Rad Mississauga, Ontario, Canada). Sample absorbance greater than 2.1 is considered positive.

High levels of GGT indicated hepatocyte injury that could be correlated to the development of hepatocellular carcinoma in the chronically infected woodchucks.[4,6] Control woodchucks had GGT levels of 5.50 ± 0.5 U/l (mean ± standard error), whereas those in infected animals were 208.8 ± 48.9 U/L, confirming that chronically infected woodchucks with hepatocellular carcinoma have greater hepatocyte injury than uninfected control woodchucks ($P < 0.002$; Figure 2). Control woodchucks had an average serum AST level of 29.3 ± 2.99 U/l, whereas that of infected woodchucks was 88.2 ± 19.2U/l ($P < 0.006$), confirming

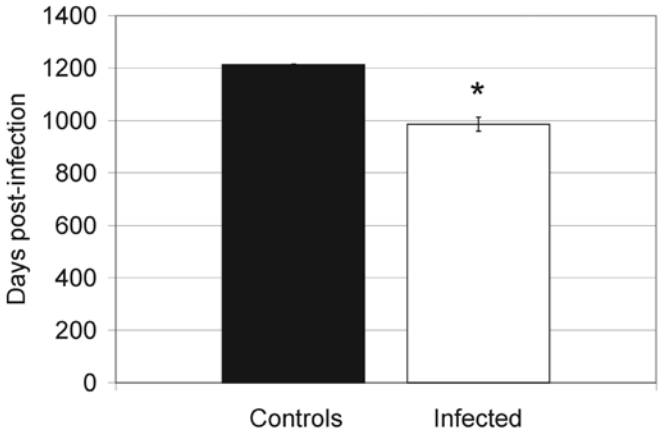

Figure 3. Average survival of woodchucks, indicating that control woodchucks (without tumors, solid bar) survive significantly (*, $P < 0.001$) longer than do those infected with woodchuck hepatitis virus (open bar). Error bar, standard error.

hepatocyte injury due to tumor growth. Chronically infected woodchucks lived for an average of 986 d ± 27 d after infection. Uninfected control woodchucks survived over the same time period without development of hepatocellular carcinoma (Figure 3).

Discussion

Isoflurane was used as an anesthetic because it provides deep-plane anesthesia with better control and faster recovery than injectable sedatives such as ketamine–xylazine[1-4,10,11] and sodium pentobarbital.[7,8,15] Premedication prior to imaging was unnecessary, due to the use of the induction chamber. Chronic experiments on the same animals would require monthly intramuscular injections of ketamine–xylazine, which can lead to permanent damage to muscle tissue and potentially tolerance to the drugs used.[2] In addition, the woodchucks were placed in the center of a 4-m MRI system for periods as long as 2 h; repeated intramuscular injections during imaging or 2 m of intravenous tubing would not be feasible. In light of the already decreased liver function of infected woodchucks, halothane was not used due to its toxicity to the liver. We found that bimonthly blood draws increase the likelihood of scar tissue in the hind paws, resulting in poor blood collection.

Tumor growth due to chronic WHV infection was monitored by MRI of the same infected woodchucks over a 6-mo period and comparing the changes to the livers with those of control woodchucks. Tumors could be identified in the images of the woodchuck liver as intense, heterogeneous regions of increased blood flow and irregular tissue margins, relative to those of control woodchucks (Figure 4). Because MRI is noninvasive, fewer woodchucks are needed (thus reducing the total number of animals required for a multiyear project), repeated invasive surgical procedures and lengthy recovery times are unnecessary to monitor tumor growth, and tumor growth occurs as it would in nature. Changes in GGT and AST, viral load, tumor growth, weight, and behavior can be monitored to better characterize the disease progress in individual animals. Compared with ultrasonography, MRI allows for better characterization of tissues changes within the entire liver. Unlike computed tomography, MRI does not use x-rays to acquire images. Monthly computed tomography would pose a risk to the animals from frequent exposure to radiation. To date, there has not been any permanent effect on

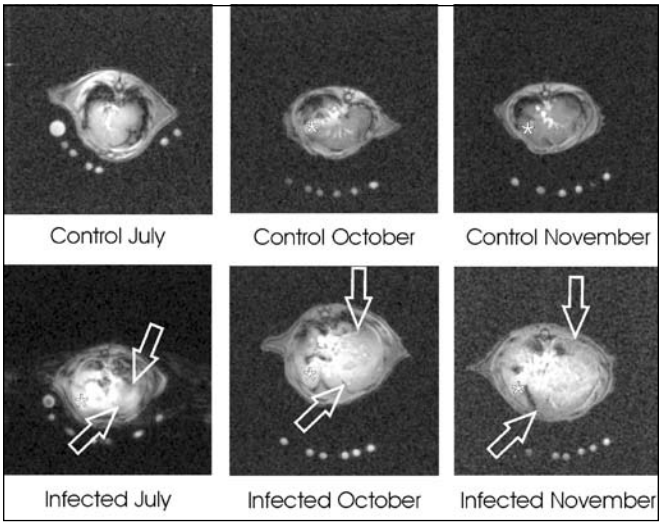

Figure 4. MR images (axial plane) of the livers of a control uninfected woodchuck (upper panels) versus an animal chronically infected with woodchuck hepatitis virus (lower panels). The edges of the tumor in the infected animal are marked with white arrows. The gall bladder is indicated by a white asterisk.

the woodchucks due to repeated MRI or lengthy anesthesia times. Control woodchucks have not developed liver tumors and are still alive. Over time, the woodchucks became accustomed to the imaging sessions and did not resist capture in the induction chamber.

Acknowledgements

The authors would like to recognize the important contributions of IBD Animal Resources for the excellent care of the woodchucks, and Thomas I Michalak, Norma D Churchill, and Colleen L Trelegan (Molecular Virology and Hepatology Research, Faculty of Medicine, Memorial University, St John's, Newfoundland) for their expertise and advice regarding this animal model.

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