NEEDLE BIOPSY FOR HEPATIC VITAMIN A LEVELS IN LIONS (PANTHERA LEO)


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Abstract: Hypovitaminosis A (HA)-related skull malformations resulting in neurologic abnormalities and death have been, and still are, reported in captive lions (Panthera leo) worldwide. Liver vitamin A (VA) concentration is the most reliable indicator of animals’ VA status, and its assessment is essential in prevention and treatment of HA in lions. A percutaneous needle liver biopsy using high-performance liquid chromatography ultraviolet retinoid analysis for VA concentration measurement was validated. It was first assessed in vitro using chicken liver. Later, the safety and feasibility of ultrasound-guided percutaneous needle liver biopsy was assessed in living lions. Hepatic VA concentrations in lion liver were measured using the above laboratory method. Mean chicken hepatic VA concentration in needle biopsy (NB) and wedge biopsy (WB) of the same liver lobes were 108.66 and 60.89 µg/g wet tissue, respectively, and were significantly (P = 0.03) correlated (r = 0.74). The calculated linear regression for predicting VA concentration in WB using NB VA for chicken liver was 25.194 + 0.3234x NB (µg/g). Four ultrasound-guided percutaneous needle liver biopsies were obtained from each of the four lions under general anesthesia. Mean hepatic VA concentration was 8.25 µg/g wet tissue (range 1.43–25.29 µg/g). Mean serum VA concentration, measured in these four lions was 1,011.1 nmol/L with a standard deviation of 337.91 nmol/L (range 590.26–1,077.2 nmol/L). The lions recovered uneventfully, and no complications were observed during a 4-yr follow-up period. In conclusion, the percutaneous needle liver biopsy technique is a reliable, practical, safe tool for obtaining liver tissue samples antemortem for assessment of the VA status in lions and can be used in future studies.

Key words: Vitamin A, lion, liver, needle biopsy, Panthera leo.

INTRODUCTION

Vitamin A (VA) is an essential fat-soluble vitamin in vertebrates, available as preformed VA (retinol) and as pro-VA (i.e., carotenoids). Carotenoids are produced and stored by plants that serve as the VA source for herbivores, and are later converted to retinol, mostly in the intestinal mucosa, and to a lesser extent, in the liver of herbivores. Retinol is stored in the liver (50–80%), kidneys, and lung and adipose tissues of the living animal, and as such, serves as the main dietary VA source for carnivores. Felids require preformed VA, in its final active form (i.e., retinol) because of their inability to convert carotenones to retinol, due to absence of the enzyme 15-15-dioxygenase, necessary for carotenoid (primarily b-carotene) cleavage. Because the liver is the major storage organ of retinol in herbivores, consumption of their internal organs as well as their muscle tissue provides wild lions (Panthera leo) with sufficient retinol. In captivity, an all-red-meat diet, deficient in internal organs, combined with uncertainties of the dietary concentration and stability of natural dietary vitamins, may lead to VA deficiency in unsupplemented meat. The daily VA requirement of lions is currently unknown. However, in domestic cats, the Association of American Feed Control Officials’ dietary VA recommendations for growth or reproduction and maintenance are 9,000 and 5,000 IU/kg of dry matter, respectively. Retinol and its metabolite, retinoic acid (RA), are essential for normal embryonic development, as well as for bone formation and remodeling, normal vision, immune functions, gene expression, and reproduction. Skull malformations,
possibly due to VA deficiency, resulting in neurologic abnormalities and death were and still are reported in captive lion cubs and young adults in many zoological gardens worldwide.\textsuperscript{2,3,4,6,15,22,26,27,33} Although circumstantial evidence suggest that VA deficiency plays the main role in the pathogenesis of skull malformations and concurrent neurologic abnormalities in captive lion populations, conclusive reports have yet to prove its existence. In addition, genetic variation may also play a role in the predisposition of certain families of lions to skull malformations, with or without association to the availability of dietary VA. Moreover, the reference range of hepatic VA level in healthy lions is based on a single wild lion only, measured in the 1970s.\textsuperscript{3} Prior to establishment of hypovitaminosis A and initiation of specific therapy, more reliable information on the normal concentration of hepatic VA and the dietary requirement of VA in healthy lions is needed. It has been reported that early VA supplementation during the course of hypovitaminosis A may lead to resolution of all clinical signs. However, when clinical signs are severe and advanced, conservative treatment may no longer be effective.\textsuperscript{6,15,33} Because early, antemortem assessment of the VA status in captive lions is considered crucial for the prevention and early treatment of hypovitaminosis A–associated disorders, an accurate, feasible, and safe assessment method is warranted.

VA levels can be measured in serum or in the liver. Although it is easy and relatively simple to measure serum VA concentration, there is controversy concerning its association with liver VA concentration, as well as to the validity of using serum VA concentration to estimate hepatic VA stores.\textsuperscript{33} Because most of the body VA stores are hepatic, the concentration of VA in the liver is considered the most accurate and reliable assessment of an animal’s VA status.\textsuperscript{1,13}

Liver VA levels in animals are measured in tissue biopsy specimens, mostly obtained through laparotomy. This is an invasive technique that requires advanced surgical skills and relatively long general anesthesia, and has potential adverse effects and complications associated with a prolonged recovery period and surgical complications, especially in wild animal species.

A reliable, safe, and short procedure to obtain liver biopsies and measure VA concentration will be beneficial, and aid in assessing, preventing, and treating of hypovitaminosis A in captive lions. The aims of this study were to validate a laboratory method for measurement of hepatic VA concentration using high performance liquid chromatography (HPLC) ultraviolet (UV) retinoid analysis in small tissue samples, obtained by needle biopsy as performed in vitro in chicken liver biopsies, and to assess the safety and feasibility of an ultrasound-guided percutaneous liver biopsy (PCNLB) technique in living lions and measure hepatic VA concentration in such samples.

**MATERIALS AND METHODS**

**Determination of VA concentration in chicken liver**

Four needle biopsies, pooled to reach a total weight of 0.03 to 0.17 g, using a 14-gauge needle biopsy gun (PRO-MAG 2.2., Manan Medical, Northbrook, Illinois, USA) and one wedge tissue biopsy, were obtained from the same liver lobe, collected from 43 fresh chicken livers obtained through a slaughterhouse. Hepatic VA was extracted from these liver samples using hexane (2 ml/sample) after adding absolute ethanol (99.9%, 1.5 and 1 ml per wedge sample and pooled needle biopsy samples, respectively) and saponification with 50% potassium hydroxide in distilled water (0.4 and 0.2 ml per wedge and pooled needle biopsy sample, respectively). Retinol-acetate was used as internal standard (2.0 and 0.4 μg per wedge and pooled needle biopsy, respectively). Absolute methanol (99.9%, 400 μl) was added after drying under N\textsubscript{2} flow. VA concentration was measured using HPLC equipped with a multi-wavelength detector (MD-910, JASCO, Tokyo, 192-8537, Japan) and a 100-mm C18-RP column (Merck, Darmstadt, 64293 Germany) using 1% acetic acid-methanol as the mobile phase.

**Determination of VA concentration in lion liver**

PCNLBs were obtained under general anesthesia from two male and two female lions ranging in age from 1.3 to 10 yr. One male and two females were from the Safari Zoological Garden, Ramat Gan, and one male lion was from the Chai Kef Zoological Garden, Rishon Lezion. The lions in both parks were fed primarily chicken with the occasional addition of whole animal carcasses that died (Safari park) or imported frozen red meat (Chai Kef Zoological Garden in Rishon Lezion). PCNLBs were obtained from the same liver lobe, collected from 43 fresh chicken livers obtained through a slaughterhouse. Hepatic VA was extracted from these liver samples using hexane (2 ml/sample) after adding absolute ethanol (99.9%, 1.5 and 1 ml per wedge sample and pooled needle biopsy samples, respectively) and saponification with 50% potassium hydroxide in distilled water (0.4 and 0.2 ml per wedge and pooled needle biopsy sample, respectively). Absolute methanol (99.9%, 400 μl) was added after drying under N\textsubscript{2} flow. VA concentration was measured using HPLC equipped with a multi-wavelength detector (MD-910, JASCO, Tokyo, 192-8537, Japan) and a 100-mm C18-RP column (Merck, Darmstadt, 64293 Germany) using 1% acetic acid-methanol as the mobile phase.
Espoo, FI-02101, Finland; 0.16 mg/kg), midazolam (Midolam, Rafa Laboratories, Jerusalem, 91003, Israel; 0.3 mg/kg) and ketamine-HCl (Ketaset, Fort Dodge, 50501, Iowa, USA; 3 mg/kg) administered i.m. by blow darts; supplementa-
tion with propofol (Diprofol, Taro Pharmaceutical
Industries, Haifa, 26247, Israel; 0.3 mg/kg) and diazepam (Assival, Teva, Debrecen, H-4042, Hun-
gary; 0.2 mg/kg) i.v.; maintenance with inhalant
isoflurane (Isoflurane, Rhodia Organique Fine,
Avonmouth, Bristol, BS11 9YF, UK; 1.5–2.5%) in
100% oxygen (2 L/min). An area of 10 cm² on the
right flank, caudal to the last rib, was clipped and
aseptically prepared. Five ultrasound (Aquila,
Esaote, Maastricht, NL 6218 GB, Holland)—guide-
ded percutaneous 14-gauge needle biopsies using a
biopsy gun (PRO-MAG 2.2., Manan Medical,
Northbrook, 60062, Illinois, USA) were obtained
from each lion through a single 1–2-cm skin
incision made immediately caudal to the last rib
halfway between the spine and the ventral midline.
The biopsy samples were taken at different angles
from the same liver lobe. Four samples were
pooled and kept on ice for 60 min, then stored at
−80°C until further analysis. The fifth needle
biopsy obtained was used for routine histology to
verify that it contained liver tissue. The skin
incision was then sutured in an intradermal
interrupted pattern using 2–0 poly(-pdioxanone)
(AssuCryl, Assut Medical, 1009 Pully-Lausanne,
Switzerland).

Blood samples for measurement of serum VA
concentration were collected from each lion
through cephalic venipuncture, placed in plain
tubes, and allowed to clot for 1 hr. Sera were then
separated by centrifugation at 3,000 g for 7 min
(EBA 20, Hettich, 532 Germany) and stored at
−20°C pending analysis. Serum VA was extracted
using 0.1 ml serum mixed with 0.2 ml absolute
ethanol (99.9%) and 1.0 ml hexane; retinol-acetate
(0.2 µg) was added to all samples as an internal
standard. The hexane phase was collected, dried
under N₂, and dissolved in 0.4 ml absolute
methanol (99.9%). Remaining VA analysis con-
tinued as previously described.

Lions were returned to the zoological garden
after the procedure and were monitored by their
caregiver for the first 24 hr, followed by a daily
evaluation by the zoo veterinarians for three
additional days. The long-term follow-up evalua-
tion of their status was performed routinely by the
zoo veterinarians over the next 48 mo. Telephone
communications with the zoo veterinarians were
made at the end of the follow-up period.

**Statistical methods**

Pearson’s correlation coefficient was calculated
in order to assess the strength of the linear
association between VA levels in the correspond-
ing chicken liver pooled-needle and wedge biopsy
samples. In addition, a linear regression model
was applied in order to estimate liver VA con-
centration from its concentration in pooled needle
biopsy samples. For both the correlation coeffi-
cient and the regression model, P < 0.05 was
considered statistically significant.

**RESULTS**

**Chicken-liver VA concentration in needle and
wedge biopsies obtained in vitro**

Liver VA concentrations in the 43 pooled
needle biopsies and corresponding 43 wedge
biopsies were measured in fresh chicken liver.
The mean weights of pooled-needle and wedge
biopsy samples were 0.110 ± 0.039 g and 0.227 ±
0.06 g, respectively. The mean pooled-needle and
wedge biopsy VA concentrations were 108.66 ±
63.49 µg/g wet tissue (ranged 33.44–257.35 µg/g)
and 60.89 ± 26.38 µg/g wet tissue (ranged 24.98–
148.53 µg/g), respectively. The mean VA concen-
tration found in the pooled needle biopsy samples
was 77% (108.66 > 60.89) higher than the mean
concentration found in the wedge biopsy samples.
There was a significant (P = 0.03) correlation
(Pearson’s r = 0.74) between liver VA concentra-
tions in the pooled needle biopsies and their
corresponding wedge biopsies (Fig. 1). The fol-
lowing linear regression model was calculated for
predicting hepatic wedge biopsy VA concentra-
tion, based on its pooled needle biopsies concen-
tration (Fig. 1): liver wedge biopsy VA concentra-
tion (µg/g wet tissue) = 25.194 + 0.3234x liver pooled
needle biopsies VA concentration (µg/g wet tissue).

**Recovery from anesthesia and long-term follow-up of the lions**

All four lions recovered uneventfully from
anesthesia and were then released into their
habitual environment on the same day. No
abnormalities in their health or performance were
recorded during a follow-up period of 48 mo.

**Hepatic and serum VA levels in living captive lions**

The signalment and serum and hepatic VA
concentrations of the lions are presented in Table
1. The weight range of the needle biopsies
obtained through PCNLB from each lion was
0.1 to 0.5 g/four biopsies. The mean hepatic VA concentration measured in the pooled needle biopsy samples was 8.53 ± 11.34 μg/g wet tissue. The mean serum VA concentrations was 1011.1 ± 337.91 nmol/L. Histology of the remaining fifth hepatic needle biopsy from each lion was confirmed to consist of liver tissue.

**DISCUSSION**

VA deficiency has been previously recorded worldwide in captive felids and particularly in lions.²,³,⁶,¹⁰,¹⁵,¹⁶,²⁷ It has been associated since the 1960s with stillbirth, weak newborn cubs, and neonatal death.¹⁶,²⁷

Hepatic VA levels in affected lions were determined only postmortem and were found to be markedly low (<0.15–48 μg/g wet tissue) compared to the level measured in a single wild lion liver (1,621.6 μg/g wet tissue).²,³,⁶ Although some lions with minor neurological dysfunction have been reported to improve following VA supplementation, their liver VA levels have never been assessed.³,¹⁵ Hepatic VA concentrations are considered the most reliable indicator of VA status both in animals and humans.⁹,²⁴ It has been demonstrated in humans that marginal depletion of VA will first cause damage to peripheral tissue before changes in serum VA concentration occur. Serum retinol concentration is tightly regulated if liver VA stores are within the physiologic range (20–300 μg/liver, in humans). Normal serum levels of VA were recorded in lions with neurologic abnormalities and occipital bone malformation that were found to have very low VA concentration in the liver.²⁴ Therefore, serum VA concentration reflects the VA storage status only when extreme depletion or overconsumption of the vitamin occurs. Hence, the measurement of serum VA provides no relevant information of the adequacy of liver VA reserves for decision-making regarding the need of substitution therapy.⁸,¹⁰,¹³

VA concentration in the liver can be measured directly in tissue samples or indirectly through measuring VA-related proteins in the blood. Retinoid acid quantification using the direct

**Table 1.** Signalment and vitamin A concentration in serum and four pooled percutaneous needle biopsy samples in four living lions (*Panthera leo*).

<table>
<thead>
<tr>
<th>Lion</th>
<th>Serum vitamin A (nmol/L)</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Pooled liver needle-biopsy sample vitamin A (μg/g wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,077.0</td>
<td>Male</td>
<td>1.16</td>
<td>5.65</td>
</tr>
<tr>
<td>2</td>
<td>1,395.1</td>
<td>Male</td>
<td>1.33</td>
<td>25.29</td>
</tr>
<tr>
<td>3</td>
<td>590.3</td>
<td>Female</td>
<td>10</td>
<td>1.74</td>
</tr>
<tr>
<td>4</td>
<td>966.9</td>
<td>Female</td>
<td>2</td>
<td>1.43</td>
</tr>
<tr>
<td>Mean</td>
<td>1,011.1</td>
<td></td>
<td></td>
<td>8.53</td>
</tr>
<tr>
<td>SD</td>
<td>337.9</td>
<td></td>
<td></td>
<td>11.34</td>
</tr>
</tbody>
</table>
HPLC-UV analysis on liver biopsies is considered the most reliable analytic method in both humans and mice. Direct HPLC/UV method was used in the present study on needle biopsies sized between 30 and 170 mg obtained through needle biopsies of the liver. This assay was proved accurate for both a wedge tissue biopsy and samples as small as 10–20 mg tissue.

In zoo and wildlife medicine, less-invasive diagnostic techniques are always preferred, and in certain cases are considered essential to allow short recovery periods and to minimize the stress of human contact. Thus, the PCNLB technique used to obtain needle biopsies seems to be most appropriate for lions. The validity of this technique was evaluated by comparing VA concentrations in paired chicken-liver wedge and pooled needle-biopsy samples and assessing their correlation. The chicken-liver tissue VA concentrations measured presently are similar to previously reported values. The PCNLB technique, when used in living wild animals, utilizes small liver tissue samples in order to minimize adverse effects and complications such as bleeding or peritonitis. Another possible advantage of the needle biopsy technique is that it enables sampling liver tissue from deeper layers and additionally, a small core of tissue of 1–3-cm length is acquired, which may better represent the overall VA concentration in different liver areas. On the other hand, the small sample size available by needle biopsy limits the VA assay, and may result in inaccurate measurements for several reasons. First, the hepatic VA distribution is heterogeneous, and a single small liver biopsy sample may not adequately represent its overall liver concentration. Second, the techniques used in most analytic laboratories are calibrated for VA measurement in significantly larger tissue samples, necessitating special calibration for small tissue samples such as in needle biopsies.

In order to overcome the limitation of the small tissue sample size of a needle biopsy, four biopsies obtained from different angles from the same liver lobe were pooled, both in chicken liver in vitro and in living lions. This pooling method is likely to yield a more representative liver tissue sample for evaluation of the overall VA status of the animal, but may still be different compared to a single, larger, albeit superficial, wedge biopsy obtained through surgery from the edge of a single liver lobe. Such differences of liver tissue sampled by these methods may account in part for the moderate result of the correlation between VA concentrations in the two sampling methods obtained in vitro. Deeper liver tissue may have higher VA concentration compared to superficial tissue samples, and thus, a needle biopsy is likely expected to yield higher VA concentration compared to a wedge biopsy obtained from the edge of the same liver lobe. A better correlation between VA concentrations measured in micro (needle biopsies) and macro (wedge biopsies) liver biopsy samples has been reported using the HPLC-UV method in samples obtained from humans at necropsy. In both the previous study in human livers as well as in the present study, VA concentrations measured in the needle biopsies are higher compared to that in the wedge samples. This consistent finding might partially result from the effects of increased UV- and other wavelength-absorbing contaminants introduced by solvents and tubing. These may have relatively greater effects on the measured VA values when small tissue samples are processed.

Further studies in lions, measuring VA concentration in four pooled liver needle biopsies, as presently described, and in a paired wedge liver biopsies from the same liver lobes, and assessing their correlation are warranted to form a linear regression formula that will be applicable to lions. Such formula can then be used to approximate VA concentration of pooled liver needle biopsies to a single surgical liver wedge biopsy. Based on preliminary in vitro studies utilizing chicken liver as a model (Shamir, unpubl. data), improved accuracy using the PCNLB technique is achieved if at least four samples are obtained per liver and pooled. These should be sampled through the same skin incision and from the same liver lobe, to minimize risks and complications, however, at different puncture angles to achieve a better general representation of this liver lobe. The chicken liver VA concentration in the needle biopsy samples was consistently higher compared to that of the corresponding wedge biopsy samples, and was in agreement with previous research on human livers. Thus, it may be assumed that when VA concentration measured in pooled needle biopsy samples from the liver of a living animal are below reference intervals, a true state of hypovitaminosis A is probably present.

The hepatic VA concentrations measured in the four lions of this present study are considerably lower compared to a previously recorded concentration in a single healthy lion from the wild as well its mean concentration measured in a group of free-ranging Florida panthers (376.88 μg/g wet tissue, ranging from 46.48 to 952 μg/g wet tissue), but similar to the results in lions that died or were
surgically treated following neurologic deterioration, presumably due to hypovitaminosis A.\textsuperscript{1,10,24,35} In addition, the hepatic VA concentrations in the lions of this present study are lower than the documented adequate hepatic VA concentrations in most carnivores, ranging from 84 to 280 μg/g wet tissue, and for those reported in domestic animal species (42 to 280 μg/g wet weight).\textsuperscript{24} Serum VA concentration measured herein in lions is within the serum reference ranges reported in most mammals, which range from 700 to 1,700 nmol/L as well as in a group of 60 free-ranging Florida panthers (350–1,300 nmol/L).\textsuperscript{10,24,25,29} As no reference interval for serum or hepatic VA have been reported in lions, it should be established. The latter should be based on a considerably larger number of healthy animals and should be established using the needle biopsy technique as the sampling method before this technique can be applied to evaluate clinical cases and before a true state of VA deficiency can be declared. Based on the present data, such a future study can be safely done using the PCNLB technique described herein.

The main limitation of the PCNLB method is the small amount of liver tissue obtained, which might adversely affect the measurement accuracy of the extracted retinol concentration. A correlation of concentrations measured using this method with wedge biopsy VA concentration must be determined in lions as part of a validation of the technique. The limited number of available lions presently made it impossible to draw general conclusions regarding hepatic VA concentration of lions in captivity. Nevertheless, liver samples were safely obtained from living lions, using the PCNLB technique under a short, 30-min general anesthesia, and utilized for hepatic VA concentration. Although the number of lions is presently limited, the study has shown that the procedure is safe and feasible, as no complications were observed over a 4-yr follow-up period. This technique is described herein for the first time in lions.

**CONCLUSIONS**

The authors believe that PCNLB is a reliable and practical tool for obtaining liver tissue samples antemortem for assessment of VA status in lions. The presently described method can be used in future studies, in both captive and free-living lions. These preliminary findings warrant further investigations of liver VA concentrations in healthy, captive, and wild lions as well as the distribution in liver VA concentrations at different depths and in different liver lobes. Because skull malformations occur during the fetal and early life periods, VA concentration should also be measured in pregnant female lions, aborted fetuses, and neonatal cubs.\textsuperscript{11,17}

**LITERATURE CITED**


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