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Correlations relating plasma vitamin D, plasma biomarkers of oxidative stress, and day-of-year in distressed immature California sea lions (*Zalophus californianus*)

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Lay summary

Upon environmental challenge, cells produce excess toxic free radicals resulting in oxidative stress, a condition partially ameliorated by vitamin D. Testing blood samples from young distressed California sea lions revealed that vitamin D status correlated negatively with oxidative stress, and both free vitamin D and oxidation radicals decreased during the transition from winter to summer.

Abstract

Fifty-two plasma samples were obtained from immature California sea lions (*Zalophus californianus*) under care at the Pacific Marine Mammal Center, Laguna Beach, CA. They arrived between January and July in the years 2010 to 2013, and presented with a variety of diagnoses, but predominantly starvation or domoic acid toxicosis. Plasma chemistry, 25-hydroxycholecalciferol [25-(OH)D_3] and biomarkers of oxidative stress (reactive oxygen species [ROS], antioxidant activity and uric acid) were tested. Because reference intervals for most of these parameters are not available, correlations were used as a basis for inference. Low to moderate correlations for 25-(OH)D_3 included: Positive with respect to antioxidant levels and calcium/phosphorus ratio; negative with respect to phosphorus and the ratio of ROS to antioxidants (a measure of overall oxidative stress). There was evidence that the vitamin D status and the burden of ROS decreased during the transition from winter to summer. Clinical and environmental implications are discussed.

Introduction

Vitamin D, a secosteroid hormone having functions long-attributed to bone and calcium metabolism (DeLuca, 1979), has emerged with pleiotropic autocrine, paracrine and endocrine effects on the kidneys, heart, and immune systems (Hewison 2012; Lai & Fang 2013). Indeed, the nuclear vitamin D receptor (VDR) has been identified in over 30 tissues in humans (Norman 2006), and there are thousands of genomic VDR binding sites in each one (Carlberg 2014). Aside from its classical nuclear location, the VDR also is present in mitochondria (Silvagno et al 2010) where it modulates oxidative function (Bouillon & Verstuy 2013).

The bounty of ATP generated from oxygen metabolism is burdened by the production of reactive oxygen species (ROS) that are capable of oxidizing vital cellular components (Finkel & Holbrook 2000). In response, animals have evolved a diversity of molecular and structural antioxidant defenses along with reduction/oxidation signaling pathways (Pamploma & Costantini 2011). The balance between ROS and antioxidants is dynamic; the biochemical effect of the incomplete quenching of ROS is termed oxidative stress (OS) (Finkel & Holbrook 2000). The transcription factor NF-E2-related factor 2 (Nrf2) is a crucial gate-keeper in this protective response. Upon challenge by accumulated ROS, it localizes to the nucleus where it upregulates the genes encoding many cytoprotective responses including antioxidant networks (Li & Kong 2009; Surh et al 2008). Vitamin D plays a role in this process, suppressing systemic oxidative stress by direct interaction with Nrf2 (Nakai et al 2013).

In human medicine, studies in the field of critical illness have been undertaken on oxidative stress (Gutteridge & Mitchell 1999) and vitamin D deficiency (Lee 2011), with suggestions that improvement of clinical outcomes might follow from appropriately-designed supplementation. Moreover, Codoner-French, et al (2012) found that low vitamin D status was associated with increased markers for OS and inflammation in obese children. The purpose of the present study is to explore the interrelation-ships of vitamin D status, specifically serum 25-hydroxycholecalciferol [25-(OH)D_3], biomarkers of OS, and day-of-year in young distressed California sea lions (*Zalophus californianus*) at the Pacific Marine Mammal Center, Laguna Beach, California.

Materials and methods

Seventy-eight plasma samples were analyzed after being obtained from distressed California sea lions admitted to the Center from 2010 to 2013. Immature animals represented the largest age cohort (52 animals) and their data were used for this study. Blood samples were collected from the jugular vein, allowed to clot, and the plasma separated. Samples not assayed immediately were stored at -34 ^{o} C. Admission dates (and blood sampling dates) spanned roughly the first seven months of the year (January 9 to July 23). The sole exception was a case admitted on 11/19/10 and retained in the data. The admitting diagnoses were: malnutrition with or without complications such as wounds or abscesses (78%); domoic acid toxicosis (14%); and miscellaneous conditions (8%). One aliquot of 42 serum samples was sent to IDEXX Laboratories (Irvine, California) for routine clinical biochemistry profiles (10 samples were not analyzed). Another aliquot (52 samples) was sent to the Michigan State University Diagnostic Center for Population and Animal Heath (Lansing, MI) for determination of 25-(OH)D_3 concentrations by radioimmunoassay (Van Saun et al 1996).

From a third aliquot (52 samples) biomarkers for oxidative stress (ROS and antioxidants) were determined using the FREE System (Diacron International, Grosseto, Italy) and its associated test kits for plasma analytes d-ROM, BAP, and uric acid. The d-ROM (reactive oxygen metabolites) test was employed to assay ROS. Using iron released from plasma proteins, reactive oxygen metabolites (primarily hydroperoxides) generate alkoxyl and peroxyl radicals via the Fenton reaction. These radicals oxidize an alkyl-substituted aromatic amine that has been dissolved in a chromogenic mixture. The resulting derivatives in the colored solution are quantified photometrically at 505 nm (Vassalle et al 2006). Results are expressed in Carratelli units (1 U CARR = 0.08 mg hydrogen peroxide/dl).

In the blood antioxidant potential (BAP) test, serum is added to a colored solution obtained by mixing ferric chloride with a thiocyanate chromogenic substrate. The decrease in the intensity of the color is measured photometrically at 505 nm. The results are expressed as μmol/L of reduced ferric ions (Benzie & Strain 1996). Both d-ROM and BAP tests have been validated in the dog (Pasquini et al 2008).

An organism's level of OS should entail direct comparison of both pro-oxidants and antioxidants (Costantini & Verhulst 2009). In the present study, BAP and d-ROM tests were correlated. Thus, following the model developed by Costantini, et al. (2006 and 2007) in wild birds, it was considered appropriate to quantify the level of OS in plasma by calculating the ratio between d-ROM and BAP (x 1000), with higher values representing higher levels of OS.

In 33 samples, plasma uric acid concentrations were determined using the FREE System. Hydrogen peroxide, produced by a uricase-catalyzed reaction of uric acid, is reacted with *p*-hydroxy-benzoate and 4-aminoantipyrine to form a colored complex. This is measured photometrically at 545nm (Trivedi et al 1978).

The ratio between calcium and phosphorus (Ca:P) was calculated due to its importance to the status of bone metabolism (Whalen et al 1977).

The day-of-year number for the date of sampling was obtained from a perpetual calendar in which each day is numbered from 1 to 365 beginning on January 1 (disc.gsfc.nasa.gov/julian_calendar.pdf).

All biomarker data were tested for conformity to a Normal distribution (Komogorov-Smirnov test). The d-ROM and d-ROM:BAP ratio (x 1000) were non-normal distributions, so their summary statistics are given as median and interquartile range (IQR); their correlations were evaluated by a nonparametric test (Spearman Rank Correlation). The other data were summarized in terms of mean and standard deviation (SD) and the correlations determined by Pearson r. Statistical calculations employed Instat® (GraphPad Software, Inc, La Jolla, CA).

Results

The summary statistics for the plasma data are presented in Table 1. OS parameters are represented by d-ROM, BAP, uric acid, and d-ROM:BAP ratio (x1000). Parameters related to vitamin D and bone metabolism are 25-(OH)D_3, calcium, phosphorus, Ca:P, and alkaline phosphatase. Compared to data reported in a study involving 10 normal wild northern elephant seal pups (*Mirounga angustirostris*) (Costa & Ortiz 1982), the sea lion mean plasma calcium was low, and the mean plasma phosphorus, and alkaline phosphatase were within the reference ranges. The Ca:P was not specified by Costa & Ortiz (1982), but some basis for comparison is afforded by the ratio of the mean values of these electrolytes. There was increased plasma uric acid compared with reference values taken from Worthy & Levigne (1982), who recorded results in small groups (3-9 animals) of fasted and fed harp seal (*Phoca groenlandica*) weaned pups. Because reference ranges were not available for 25-(OH)D_3 and the OS parameters, a basis for inference was attempted using correlation (Table 2).

Correlation statistics are presented in Table 2. Using 25-(OH)D_3 as the independent variable, low to moderate correlations, along with sufficient evidence ($p < 0.05$) to reject the null hypothesis were: positive correlations were observed between 25-(OH)D_3 and with both BAP, and Ca:P; negative correlations were observed between 25-(OH)D_3 and with both d-ROM:BAP, and phosphorus. There were negative correlations between the day-of-year and with both 25-(OH)D_3 and d-ROM. Graphs of scatterplots and regression lines are presented for 25-cholecalciferol in relation to dROM:BAPX1000 and day of year (Figures 1 & 2, respectively).

Discussion

The life histories of marine pinnipeds have required their adaptation to marked swings in oxidative stress. The physiological response to apnea and hyperoxia in deep-diving causes flares of ROS (Vazquez-Medina et al 2011a; Vazquez-Medina et al 2011b; Welker et al 2013). In northern elephant seals, prolonged fasting while breeding, molting or weaning induces OS and compensatory antioxidant production (Sonanez-Organis et al 2012; Vasquez-Medina JP 2013). Xenobiotic pollutants can also induce chronic OS (Hirakawa et al 2011). Indeed, fasting was shown to stimulate Nrf2 activity in post-weaning northern elephant seals (Vazquez-Medina et al 2013).

The sea lions in this study presented with complex problems involving stress-inducing conditions, most notably starvation that would increase ROS production (Arthur PG et al 2008). In addition to promoting antioxidant defenses via Nrf2, cytoprotective responses like autophagy would also be initiated (Scherz-Shouval et al 2007). Unfortunately for these young patients, the maximum anti-oxidant response may not be fully developed until maturity, according to a study in hooded seals (*Cystophora cristata*), (Vazquez-Medina at al 2011a). Therefore, the nonparametric distributions of the d-ROM and d-ROM:BAP ratio may be due to the underlying heterogeneity of the diseases present and the animals' responses to them. A similar finding was reported in a study of elderly humans, where ROS markers and vitamin D were examined in blood mononuclear cells (Wiley et al 2014). That study population likewise consisted of subjects with various impairments and health risks.

Uric acid, an end-product of purine metabolism, is an acknowledged plasma antioxidant (Strazzullo & Puig 2007). In weaned northern elephant seal pups, robust purine salvage and recycling is an important adaptation to fasting and the concomitant increased plasma ROS (Sonanez-Organis et al 2012). Fasted groups had non-significant increases of uric acid over the fed group. In the present sea lion study, the range of values was greater than that reported in harp seals, and may reflect a response to the challenge of OS. No correlations were found between uric acid and BAP or 25-(OH)D_3.

25-(OH)D_3 is the storage form of the vitamin and has long been the preferred test to determine overall vitamin D status (Vieth 1999; Hollis 2005). The purpose of conducting this study with one age group (immature) was to reduce the influence of growth as a variable in the vitamin D activity (Spanos et al 1978, Horst and Littledike 1982). There are 2 small studies recording plasma 25-(OH)D_3 in normal pinnipeds. Hooded seals (n=7) maintained under various conditions in captivity had mean levels of 27.5 nmol/L (Keiver et al 1988a). In the second study, the results for both captive and wild hooded, harp, and grey seals of mixed ages (total n=22) ranged from 17.5 to 167 nmol/L, and a single sample from an adult

California sea lion was recorded as 145 nmol/L (Keiver et al 1988b). The 25-(OH)D_3 test results in the present study are greater (mean 202 nmol/L) compared to earlier reports. Beyond the obvious disease conditions, this difference could reflect disparate populations, species, diet, and possibly UV light exposure. It should also be noted that our results are more in line with the reference range for domestic dogs: 6 – 214 nmol/L (Michigan State University Diagnostic Center for Population and Animal Health, Lansing MI).

The positive correlation between 25-(OH)D_3 and BAP and the negative correlation with the measure of OS (d-ROM/BAP ratio) are consistent with several *in vitro* and *in vivo* studies in humans and laboratory rodent models (Garcion et al 1999; Kallay et al 2002; Bao et al 2008; Nakai et al 2013; Sinha 2013; Uberti et al 2013). The strength and statistical significance of the vitamin D correlation with OS is greater than that with BAP (no significant correlation at all found with d-ROM). This illustrates the importance of assaying both ROS and antioxidant activity when evaluating OS (Costantini 2009).

In general, it would seem that overt vitamin D deficiency is unlikely in these animals. Indeed, the normal Ca:P and alkaline phosphatase results suggest that bone mineralization and metabolism were normal. As to the low calcium, protein-energy starvation in children has been shown to decrease intestinal calcium absorption, together with a compensatory elevation of serum parathyroid hormone and 1,25-dihydroxyvitamin D (Kerstetter et al 1998). A second study in children demonstrated that, in addition to a calcium decline, there was an even greater decline in serum phosphorus, resulting in a significant rise in the Ca:P (Freiman et al 1982). These findings are the likely basis for juvenile osteoporosis in protein-energy malnutrition (Briers et al 1975) which has also been observed in wild moose calves (*Alces alces*) (Ytrehus et al 1999).

As described in a detailed case study of protein-energy starvation in a juvenile northern elephant seal, plasma calcium can reflect effects of reduced intake or loss of albumin binding sites, while plasma phosphorus can reflect effects of acid-base imbalance and growth cessation (Lawler et al 2014). In the present sea lion report, there was no relationship of 25-(OH)D_3 to calcium levels and a negative correlation with phosphorus. Further comment on these observations must await additional research into the intricacies of Ca:P regulation, vitamin D, parathyroid hormone, and fibroblast growth factor 23 in growing sea lions (Bergwitz & Juppner 2010).

The negative correlation between the day-of-year and 25-(OH)D_3 suggests that vitamin D status declined from January to July. Mammals can meet their vitamin D requirement endogenously (photochemical transformation of 7-dehydrocholesterol to previtamin D_3 in the skin) or exogenously (food). The skin of the domestic dog and cat possesses low concentrations of 7-dehydrocholesterol, and what little is present is inadequately converted by UV light exposure (How et al 1994). Thus, these species are almost completely dependent upon exogenous sources of vitamin D. No similar physiological studies have been reported in marine mammals. However, a study involving 12 female southern elephant seals (*Marounga leonina*) found that during periods on land (breeding and moulting), the serum 25-(OH)D_3 increased, from which it was inferred that there was increased endogenous synthesis owing to greater UV exposure (Wilske & Arnbom 1996). While the immature California sea lions in the present

report may have had less exposure to UV radiation while at sea versus ashore, it would nonetheless be expected that, as the spring progressed, the increasing UV duration and intensity would have had some positive impact on plasma 25-(OH)D_3. In fact, the opposite appears to be the case. The negative correlation between the day-of-year and d-ROM would seem to suggest that ROS production ameliorated during the transition from winter to summer.

The primary component of the sea lion diet is fish, whose oil content would be their food source of vitamin D. Fish, in turn, obtain the vitamin from the food chain leading ultimately from plankton that synthesize it from UV light (Bills 1927; Bjorn and Wang 2000). Here again, one would expect increasing production of vitamin D in the food chain as the months progressed from January to July. This, however, would depend upon the life cycle of the plankton migration both in terms of depth and latitude (Drummond 1934). Unfortunately, little is known about "vitamin traffic" in the ocean ecosystem (Giovanni 2012). To add to the complexity, the warmer ocean temperatures in the northeast Pacific due to climate change have affected the trophic dynamics of both fish and zooplankton populations (Ware and Thomson 2005; Mackas at al 2007) and may have influenced the results described herein.

Taken together, the findings of this study in distressed California sea lions warrant consideration of clinical trials to evaluate supplementation with antioxidants and possible vitamin D_3 during rehabilitations that are undertaken later in a given year. In a larger context, assessment of biomarkers of oxidative stress and vitamin D status using readily available tests may reveal yet another way marine mammals can be sentinels for anthropogenic environmental change in the ocean ecosystem.

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Table 1 Plasma biomarkers relating to vitamin D status and oxidative stress

Biomarker	Units	n	Mean (SD)	Median	IQR	Reference Range
d-ROM	U CARR	52	--------	161	141	Unknown
BAP	µmol/L	52	3050 (1677)	---------	-------	Unknown
d-ROM:BAP (x1000)		52	---------	66	62	Unknown
25-(OH)D_3	nmol/L	52	202 (64)	---------	-------	Unknown
Calcium	mg/dl	42	8.5 (0.8)	---------	-------	11.6-12.4[a]
Phosphorus	mg/dl	42	7.3 (3.8)	---------	-------	6.5-7.4[a]
Ca:P	Not applicable	42	1.35 (0.46)	---------	-------	1.31-1.33[a.]
Alkaline phosphatase	IU/L	42	53 (35)	---------	-------	29-84[a]
Uric acid	mg/dl	34	5.3 (2.6)	---------	-------	0.5-2.8[b]

[a]Costa and Ortiz (1982), [b]Worthy and Lavigne (1982)

Table 2 Correlations relating to 25-(OH)D_3, biomarkers of oxidative stress, and day-of-year

X	**Y**	**Correlation coefficient (r)**	***p* value**
BAP	d-ROM	0.286	0.040
Uric acid	BAP	0.288	0.104
25-(OH)D_3	d-ROM	-0.112	0.428
25-(OH)D_3	BAP	0.274	0.049
25-(OH)D_3	d-ROM:BAP (x 1000)	-0.351	0.011
25-(OH)D_3	Calcium	0.062	0.698
25-(OH)D_3	Phosphorus	-0.321	0.038
25-(OH)D_3	Ca:P	0.313	0.044
25-(OH)D_3	Alkaline phosphatase	0.139	0.380
25-(OH)D_3	Uric acid	-0.249	0.169
Day-of-year	25-(OH)D_3	-0.326	0.020
Day-of-year	d-ROM	-0.305	0.029
Day-of-year	BAP	-0.213	0.133
Day-of-year	d-ROM:BAP (x 1000)	-0.053	0.712

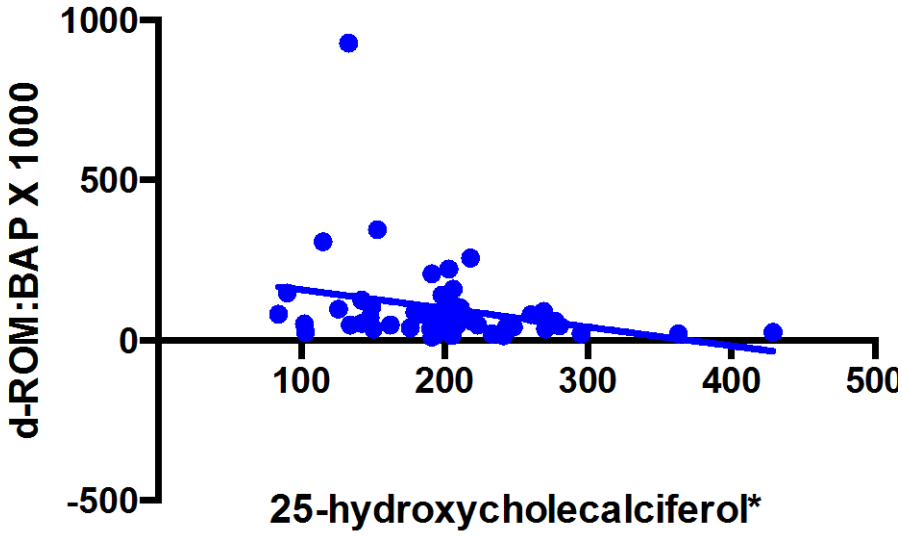

Figure 1
Scatterplot and regression line depicting the relationship between a measure of oxidative stress (d-ROM:BAP X 1000) and 25-hydroxycholecalciferol. *nmol/L

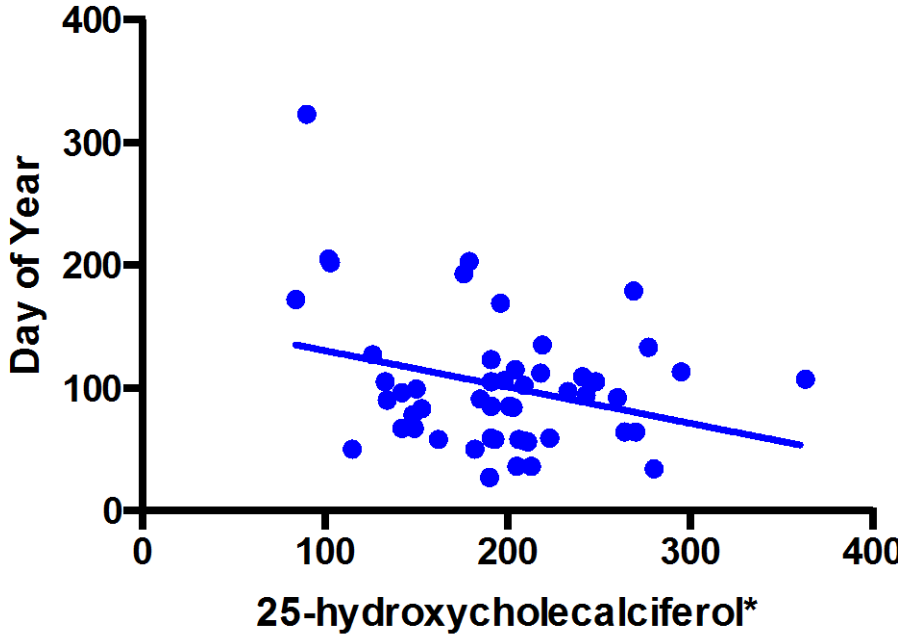

Figure 2
Scatterplot and regression line depicting the relationship between day of year of sampling and 25-hydroxycholecalciferol.*nmol/L