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Moderate maternal vitamin A deficiency alters myogenic regulatory protein expression and perinatal organ growth in the rat

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VITAMIN A IS AN ESSENTIAL nutritional factor required for a wide range of physiological processes including the promotion of general growth, maintenance of the immune and visual systems, regulation of epithelial tissues, and embryonic development. Vitamin A itself exists in three oxidation states: retinol (alcohol), retinal (aldehyde), and retinoic acid (RA). Together, these compounds are thought to carry out the various functions of vitamin A.

A high-vitamin A intake, early in pregnancy or around the time of conception, has been shown to cause birth defects (25). In addition, deficiency of this vitamin is one of the most common dietary deficiencies in the developing world and is a major health concern where it is associated with increased risk of fetal and infant mortality and morbidity (32). Unfortunately, the epidemiological data have been difficult to interpret and more mechanistic animal studies have been essential in evaluating the precise effects of vitamin A deficiency in utero.

By feeding animals a vitamin A-free diet and mating them, it has been possible to study the role of RA in embryonic development and this has revealed defects in a number of tissues/organs including the central nervous system, lungs, heart, and limbs (23). These studies, however, result in high rates of spontaneous abortion, fetal malformation, and late fetal death (30). Early experiments with moderate vitamin A deficiency, in which maternal vitamin A levels are reduced by up to 50%, have also demonstrated that, in addition to respiratory problems, neonates showed evidence of mobility problems in response to moderate vitamin A deficiency. This study investigated whether moderate deficiency of this vitamin plays a role in regulating key skeletal muscle regulatory pathways during development. Thirty female rats were fed vitamin A-moderate (VAM) or vitamin A-sufficient diets from weaning and throughout pregnancy. Fetal and neonatal hindlimb and muscle samples were collected on days 13.5, 15.5, 17.5, and 19.5 of pregnancy and 1 day following birth. Mothers fed the VAM diet had reduced retinol concentrations at all time points studied (P < 0.01), and neonates had reduced relative lung weights (P < 0.01). Fetal weight and survival did not differ between groups but neonatal survival was lower in the VAM group where neonates had increased relative heart weights (P < 0.05). Analysis of myogenic regulatory factor expression and calcineurin signaling in fetuses and neonates demonstrated decreased protein levels of myf5 [50% at 17.5 dg (P < 0.05)], myogenin [70% at birth (P < 0.001)], and myosin heavy chain fast [50% at birth (P < 0.05)] in response to moderate vitamin A deficiency. Overall, these changes suggest that vitamin A status during pregnancy may have important implications for fetal muscle development and subsequent muscle function in the offspring.

retinoic acid; calcineurin; skeletal muscle; neonate
controlling skeletal muscle development and that may explain the reduced mobility evident in the offspring.

MATERIALS AND METHODS

Animals and experimental design. Sixty female Rowett-hooded Lister rats were divided into two groups. Thirty animals were fed a vitamin A-moderate (VAM) diet and 30 animals were fed a vitamin A-sufficient (VAS) diet ad libitum from weaning (19 days, ~40 g) and throughout pregnancy. The VAM diet contained more than the recommended vitamin A content at 1,200 retinol equivalents (RE)/kg diet (3). The VAM diet contained 45 RE/kg diet. Our previous studies established that addition of 45 RE/kg diet to female rats for 30 days results in an approximate reduction in plasma retinol concentrations of up to 50% (3). This level of reduction is described as a moderate deficiency by Lelieve-Pegorier et al. (22).

These diets were prepared as described previously (3). From weaning to mating at 7 wk, the animals were group housed. At mating and throughout gestation, the animals were housed singly. These animals were not synchronized before mating, which was confirmed visually by the appearance of a vaginal plug (designated as day 0.5 of pregnancy). Pregnant dams and their fetuses were killed at 10.5, 13.5, 15.5, 17.5, and 19.5 days of gestation and one group of rats from both the VAM and VAS diets were allowed to give birth. In this group, mothers and offspring were killed 24 h after birth. Each group consisted of n = 4 to n = 7 dams. Postmortem samples of maternal, fetal, and neonatal muscles and other organs including liver, lung, and heart were dissected, weighed, and stored at −70°C. Although tissue samples were collected at 10.5 days of gestation, these were not included in the analysis, as the dissection was not regarded as accurate due to the small size; 13.5 days of gestation samples consisted of decapitated bodies with all major internal organs removed. Hindlimbs were collected at 15.5, 17.5, and 19.5 days of gestation, and muscle bulks were dissected from hindlimbs 1 day after birth. Muscle bulks comprised soleus, plantaris, and gastrocnemius muscles. All fetuses and neonates (dead and live) were counted and weighed. Maternal blood samples were obtained from the body cavity following exsanguination. The blood was centrifuged (1,000 g, 10 min, 4°C) and the plasma was stored at −70°C for retinol analysis. All experimental procedures were approved by the appropriate ethical committee and conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Determination of neonatal muscle for protein, RNA, and DNA composition. Muscle bulks from 1-day-old neonates were used for estimations of total protein, RNA, and DNA. Approximately 100 mg of muscle tissue were homogenized in 2 ml of ice-cold PCA (0.2 M) (BDH Chemicals). The homogenate was left on ice to precipitate for 30 min and then centrifuged at 2,500 rpm for 10 min. The pellet was resuspended in 5 ml of 0.3 M NaOH (Sigma, Poole, UK). The sample was then incubated at 37°C for 1 h with intermittent mixing. From the solubilized residue, 0.5 ml were removed for protein determination using the BCA protein assay (Perbio Science UK, Tattenhall, UK). One milliliter of 2 M PCA was added to the remaining 4.5 ml, which were mixed and placed on ice for 10 min. After centrifugation at 2,500 rpm for 15 min, the supernatant was removed and an RNA assay was carried out using the method of Munro and Fleck (24). To the remaining pellet, 3 ml of 0.6 M PCA were added and this was incubated at 70°C for 30 min before centrifugation at 2,500 rpm for 15 min. Three milliliters of the supernatant were then removed for DNA assay using the Burton method (8).

Western immunoblotting analysis. Fetal samples from each of the time points were selected from dams fed the VAM or VAS diets (n = 3 each). These samples were taken from ~70°C and homogenized (Polytron, Kinematica) on ice for 30 s in 0.5 ml of Sorbitol-Tris-EDTA buffer (STE) (20 mM Tris, pH 7.5, 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 1 μg/ml leupeptin, 2 μg/ml aproatin, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A). After incubation on ice for 1 h with intermittent mixing, the homogenate was spun at 100,000 g for 1 h at 4°C, and the supernatant, designated the cytosolic fraction, was removed. The pellet was resuspended in the above buffer with the addition of 1% Triton X-100 and incubated on ice with intermittent mixing for 1 h.

The homogenate was centrifuged at 100,000 g for 30 min at 4°C, and the supernatant, designated the membrane fraction, was removed and stored. Protein determination was carried out using the BCA protein assay (Perbio Science UK). Equal amounts (50 μg) of cytosolic and membrane fraction were separated by SDS-PAGE and electrotansferred onto Hybond-C membrane (Amersham, Little Chalfont, UK). The membrane was blocked in Tris-buffered saline (TBS; TBST, pH 7.6, with 0.1% Tween 20) containing 5% (wt/vol) dried milk (Marvel, Stafford, UK). Primary antibodies were added at the manufacturer’s recommended dilution in TBST + 5% dried milk, and after being washed, membranes were then incubated in secondary antibody conjugated to horseradish peroxidase at 1:2,000 dilution. After further washing, the membranes were subjected to chemiluminescent detection (Perbio Science UK). The specificity of the bands was assessed by molecular mass and the inclusion of an appropriate positive control from the manufacturer. Autoradiograms were scanned (Epson GT-9500) and individual protein bands were quantified using the Phoretix 1D Advanced analysis package Version 4.0. (Phoretix International, Newcastle-upon-Tyne, UK). All primary antibodies were purchased from Santa Cruz Biotechnology (Autogen Bioclear UK, Calne, UK) with the exception of MHC-fast (Novocastra Laboratories, Newcastle upon Tyne, UK). Secondary antibodies were goat anti-mouse (DAKO, Cambridge, UK) and goat anti-rabbit (Chemicon Europe, Chanders Ford, UK). Equivalent total protein loading on membranes was confirmed by staining in Poncaev Red stain (Sigma).

Retinol analysis. Maternal plasma retinol levels were determined by reverse-phase HPLC as described by Hess et al. (18) using echinone as an internal standard (Hoffmann-La Roche, Basle, Switzerland). All samples were analyzed in duplicate. Data were collected by Waters Millenium 32 software, and plasma retinol concentration was calculated using the formula: [Retinol] = retinal × 100/echinone.

Statistical analysis. Student’s t-test was used to test for differences in DNA, RNA, and protein between VAS and VAM samples 1 day after birth. For all other analysis, ANOVA was used as these comparisons were of more than two groups or more than one factor (e.g., day and treatment). Two-way ANOVA was used to examine differences in protein abundance from Western analysis that was carried out on samples from at least three different animals in each case. GenStat 5th Edition was applied to log data from these separate experiments. The t-statistic based on the common standard error of the difference was applied to the mean values to identify the differences. For the graphical presentations, the data were normalized by taking the 1 day and treatment. For the graphical presentations, the data were normalized by taking the 1 day of gestation neonatal VAS sample and setting its expression equal to 100%, then every other sample was expressed as a percentage of this value. The mean of each percentage ± SE was then plotted in the graphs that relate to Western analysis.

RESULTS

Moderate vitamin A deficiency on maternal and perinatal parameters. The VAM diet resulted in reduced maternal retinol concentrations that were significant at all time points (Fig. 1A). Additionally, between 10.5 days of gestation and 1-day-old neonates, there was an 85% reduction in the levels of maternal plasma retinol in the VAM-fed animals (P < 0.001).

The pregnancy rates were measured at each nominated time point. This was determined at either the time of slaughter for fetal sampling or on the day of birth, in the case of those animals that went to parturition. The rate was expressed as a ratio of the number of pregnant rats to the number of rats.
samples from the VAM group tended to be lower than that of the VAS group ($P = 0.08$), although this did not reach statistical significance. There were no differences in the total protein or total RNA in these muscle bulks (Table 1).

**Effects of moderate vitamin A deficiency on muscle protein expression.** It is important to note that the time course studied did not comprise a homogeneous sample set. The samples analyzed were eviscerated and decapitated bodies at 13.5 days of gestation, hindlimbs at 15.5–19.5 days of gestation, and muscle bulks in 1-day-old neonates. Where protein abundance was significantly affected by day of gestation, ANOVA was performed using only the 15.5–19.5 days of gestation samples. A treatment effect is only identified between samples at the same time point.

**Effect of vitamin A deficiency on proteins of the CaN pathway.** No changes in CaN protein abundance were observed in response to moderate vitamin A deficiency and abundance appeared relatively constant across the developmental window (Fig. 2). Also, total protein abundance of NFATc1 and NFATc3 proteins, downstream effectors in the CaN pathway, did not alter significantly with deficiency (Figs. 3 and 4). NFATc1 was detected as multiple bands on a Western blot, ranging from 80 to 115 kDa (Fig. 3), whereas NFATc3 was examined. In both the VAS and VAM groups, there was no change in the pregnancy rates with 90% of the animals examined being pregnant (data not shown). Fetal and neonatal number per dam was not affected by treatment either (data not shown). However, although it appeared that the VAM diet had no effect on either conception or fetal and neonatal number, the neonatal survival rate was reduced by 40% ($P < 0.01$; Fig. 1B). It was observed that neonates from the VAM group showed evidence of reduced mobility: the pups were unable to move and those that were mobile moved at a slower rate than pups from the VAS group.

There were no differences in fetal or neonatal body weights between VAS and VAM groups nor were there any differences in relative liver weights (Table 1). However, when relative lung weights were examined, VAM neonates were found to have significantly smaller (15%) lung weight ($P < 0.01$). Conversely, when the relative heart weights were measured in the neonates, the VAM group showed an increase (33%) in heart weight ($P < 0.05$; Table 1).

Biochemical analysis was also carried out on the muscle bulks from 1-day neonates. The total amount of DNA in

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### Table 1. Fetal/neonatal body weights, relative organ weights, and composition of mixed muscle from 1-day neonatal hindlimbs from mothers fed VAS and VAM diets

<table>
<thead>
<tr>
<th></th>
<th>VAS</th>
<th>VAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal weight, g</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>15.5dg ($n = 4$)</td>
<td>0.260</td>
<td>0.096</td>
</tr>
<tr>
<td>17.5dg ($n = 4$)</td>
<td>0.760</td>
<td>0.045</td>
</tr>
<tr>
<td>19.5dg ($n = 4$)</td>
<td>1.890</td>
<td>0.108</td>
</tr>
<tr>
<td>1dNeo ($n = 6$)</td>
<td>5.250</td>
<td>0.311</td>
</tr>
<tr>
<td>Relative organ weights, g/100 g body weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17.5dg</td>
<td>7.700</td>
<td>0.556</td>
</tr>
<tr>
<td>19.5dg</td>
<td>6.400</td>
<td>0.232</td>
</tr>
<tr>
<td>1dNeo</td>
<td>4.100</td>
<td>0.117</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19.5dg</td>
<td>3.100</td>
<td>0.093</td>
</tr>
<tr>
<td>1dNeo</td>
<td>2.300</td>
<td>0.057</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1dNeo</td>
<td>0.600</td>
<td>0.019</td>
</tr>
<tr>
<td>Muscle bulks, mg</td>
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<td></td>
</tr>
<tr>
<td>1dNeo</td>
<td>32.4</td>
<td>1.15</td>
</tr>
<tr>
<td>Total protein, μg</td>
<td>5,909</td>
<td>1,254</td>
</tr>
<tr>
<td>Total RNA, μg</td>
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<td>50</td>
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<tr>
<td>Total DNA, μg</td>
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</tr>
<tr>
<td>RNA/protein</td>
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<td>5</td>
</tr>
<tr>
<td>RNA/DNA</td>
<td>0.67</td>
<td>0.06</td>
</tr>
<tr>
<td>DNA/protein</td>
<td>89</td>
<td>15</td>
</tr>
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</table>

Values are means ± SE. Significant differences between the body/organ weights between the vitamin A-sufficient (VAS) and vitamin A-moderate (VAM) groups at each time point were determined by ANOVA and any differences were separated using the $t$-statistic: $P < 0.05$, $P < 0.01$. Significant differences between the biochemical parameters were determined by Student’s $t$-test: $P = 0.08$ ($n$ value refers to dam number). dg, Days of gestation; 1dNeo, 1-day-old neonates.
detected as multiple bands ranging from 75 to 120 kDa (Fig. 4). NFATc1 levels rose sharply at 17.5 days of gestation ($P < 0.01$) and then decreased at birth. Conversely, NFATc3 levels were highest at earlier time points and then decreased at 17.5 days of gestation until birth ($P < 0.001$; Figs. 3 and 4). Examination of the protein abundance of the fastest migrating band alone (arrowed) demonstrated no changes in abundance due to moderate vitamin A deficiency (not shown).

**Effect of vitamin A deficiency on MRF expression.** The levels of the MRF family of proteins (myf5, myf6, myogenin and myoD) were examined during development. Myf5 and myogenin demonstrated altered protein levels in response to moderate vitamin A deficiency. A significant decrease in myf5 abundance by 50% was detected in the VAM samples at 17.5 days of gestation ($P < 0.05$; Fig. 5). From 17.5 to 19.5 days of gestation, the myf5 protein was detected as a doublet. Myogenin levels were also reduced by 70% with moderate vitamin A deficiency following birth ($P < 0.001$; Fig. 6). MyoD and myf6 exhibited no significant differences in protein abundance due to the deficiency (data not shown).

**Effect of moderate vitamin A deficiency on skeletal muscle contractile proteins.** MHC-fast expression was barely detectable at time points before 19.5 days of gestation (Fig. 7). At this time point, moderate vitamin A deficiency resulted in an 800% increase in levels of MHC-fast ($P < 0.001$). However, MHC-fast levels were decreased by 50% in 1-day-old neonates muscle bulks ($P < 0.05$) compared with VAS controls (Fig. 7). No significant changes in the abundance of MHCdev or MHCslow proteins with moderate vitamin A deficiency were observed across these time points (data not shown).

**DISCUSSION**

Verification of the moderate vitamin A model and effects on perinatal organ development. At each of the time points studied across gestation, maternal plasma retinol concentrations were lower in the VAM group compared with those in the VAS group. This was a good indication that moderate maternal vitamin A deficiency had been achieved. In support of this finding, the relative neonatal lung weights were lower in the VAM group compared with VAS group. Moderate vitamin A deficiency has previously been shown to adversely affect lung development in particular (2, 3). In contrast, the vitamin A deficiency was not found to cause differences in total fetal or neonatal body or relative liver weights, again, in agreement with previous studies by Antipatis et al. (3) where a similar model was used. Additionally, there were no differences in fetal or neonatal number or pregnancy rates as a result of moderate vitamin A deficiency confirming previous findings (22). However, although there were no differences in fetal survival rate throughout gestation, survival of the neonates was dramatically reduced in the offspring of VAM-fed dams. This is the first study to report that even a moderate vitamin A deficiency can reduce survival rates at birth. This may be related to the reduced size and presumed impaired function of the lungs. The neonates from vitamin A-deficient rats and guinea pigs have been reported to gasp for air and die within several minutes, probably due to an inability to obtain oxygen (4). In contrast to the effects on relative lung weight, relative heart weights showed an increase in the neonates from the VAM group compared with those from the VAS group.
Effects can be attributed to RA, which is crucial for structural as well as developmental changes during development of this organ (27). This is the first study to show an increase in relative heart weight in the offspring at birth from mothers with moderate vitamin A deficiency. This result would agree with the finding of increased relative heart weight with marginal vitamin A deficiency in rats at day 18 of life by Wright et al. (34). However, a previous moderate vitamin A deficiency study (3) did not detect this increase; however, this latter study measured relative organ weights at 20 days gestation and not at birth. During development, the heart is one of the first organs to develop. Therefore, in the VAM group, heart development occurs at a time when maternal vitamin A levels are relatively high (~50% of the sufficient group). Lung development, however, occurs later in development, and at this time the maternal vitamin A levels in the VAM group had depleted (to ~10% of the sufficient group) and therefore proportionately less vitamin may have been available for fetal lung maturation. An increase in the relative heart weight in neonates from the VAM group may be indicative of a vitamin A-induced effect on cardiac muscle hyperplasia and/or hypertrophy. Interestingly, this finding is in agreement with the theory that an RA-mediated pathway is believed to repress myocardial cell hypertrophy in vitro (35).

**Effects of moderate vitamin A deficiency on muscle protein expression.** This current study was based on the hypothesis that creating an environment moderately deficient in vitamin A during pregnancy would result in the modulation of skeletal muscle development in the offspring. This was based on a number of in vitro studies in which RA has been shown to affect the function of several key regulatory proteins involved in muscle development, such as CaN and the MRFs.

In this study, moderate vitamin A deficiency had no effect on either the total weight of the hindlimbs at birth or on biochemical parameters of these limbs including their total protein, total RNA, or total DNA content. However, there was a tendency for the total DNA, in samples from the moderate deficient group, to be less than those in the sufficient group ($P = 0.08$). This may suggest a potential reduction in muscle cell hyperplasia with moderate vitamin A deficiency. In support of this finding, previous work has shown that a low nutritional diet in sheep during pregnancy resulted in a reduction of DNA of the semitendinosus muscle of the offspring at birth (17). It was also possible that a lower amount of DNA in VAM samples was indicative of a decrease in the muscle satellite cell population. However, maternal nutritional deprivation studies during rat gestation have indicated that alterations in satellite cell number only become apparent beyond 1 day postpartum (7).

As the CaN pathway plays an important role in controlling muscle growth and development, it was then investigated whether moderate vitamin A deficiency altered CaN signaling in developing hindlimbs and muscle. This study was initiated based on findings in vitro that RA can regulate CaN function, either by inhibiting CaN activity (28) or by upregulating CaN protein levels (21).

It was observed that, in this model of moderate vitamin A deficiency, there were no detectable differences in total CaN protein abundance across the developmental period from 13.5 days of gestation until birth. However, it was possible that CaN...
protein levels may have changed at time points in between sampling point(s) but were then restored to control levels. Equally, it is possible that changes in total CaN protein occurred within specific muscle/tissues/cell types and that these subtle differences were lost in our gross analysis of total hindlimb and muscle bulks. However, we also observed no changes in the total levels of the downstream NFATc1 or NFATc3 proteins, and crucially there were no obvious differences in the observed banding patterns of these proteins with vitamin A deficiency. The NFATs are known substrates of CaN, and these two isoforms are particularly abundant in skeletal muscle (19). Changes in their apparent molecular mass/bands have been associated with changes in their phosphorylation state (12) that gives an indirect measurement of CaN activity. From these results, we infer that moderate vitamin A deficiency does not appear to alter CaN activity toward these substrates. However, it cannot formally be ruled out that a change in CaN activity is apparent but that there is a compensatory change in the NFAT kinase activity such that the steady-state level of NFAT phosphorylation remains similar.

It is possible that previous in vitro results obtained on cells in culture do not extend to a developmental rat model in vivo or that a deficiency of RA does not result in an inverse response to oversupply. Alternatively, the level of vitamin A deficiency imposed may not have been severe enough to invoke alterations in CaN signaling. However, this is unlikely as differences in the expression of two of the four MRFs were detected in response to deficiency. Both myf5 and myogenin were detected at lower levels in response to moderate vitamin A deficiency. Myogenin expression was reduced in muscle from moderate vitamin A-deficient 1-day-old neonates. This reduction is consistent with studies that show that RA promotes differentiation and induces myogenin expression in rat rhabdomyosarcoma cells (5). A decrease in myogenin protein abundance at this time point may represent a reduction in the potential to form slow fibers due to moderate vitamin A deficiency, as myogenin is prevalent in slow-twitch muscles in the mature limb (20). Myf5 protein expression was also reduced in 17.5 days of gestation hindlimbs with moderate deficiency. Myf5 is known to play an important role in the survival and commitment of the myoblast phenotype (26). A reduction in myf5 abundance occurring at this time [before secondary myogenesis (33)] may indicate fewer determined, proliferating myoblasts that may contribute, potentially, to a reduction in the secondary/fast fiber cohort. This hypothesis is supported by the reduced expression of MHC-fast protein and by the tendency for total DNA to be reduced in the moderately deficient neonatal muscle. This scenario would agree with many other studies that show that the development of the slow-fiber cohort is protected during other nutritional deprivation regimes (14, 31). Changes in the fiber makeup of the muscles of the hindlimb may ultimately contribute to altered muscle function in the offspring.

The objective of the current study was to test whether moderate vitamin A deficiency affects hindlimb development in the rat by regulating CaN signaling and MRF expression. Although moderate vitamin A deficiency appeared to have no clear effect in regulating the CaN signaling pathway, changes in the abundance of MRFs and myosin structural proteins clearly suggest that muscle development was perturbed. These findings suggest that in this model system, vitamin A regulates
MRF expression independently of CaN and that vitamin A status during pregnancy may have important implications for fetal muscle development and subsequent muscle function in the offspring. In addition, it will be important to determine to what extent these effects are permanent or whether they are subsequently reversed by vitamin A repletion during later life.

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REFERENCES


