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RESEARCH ARTICLES

All-*trans* retinoic acid modifies the expression of clock and disease marker genes

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Abstract

Restricted feeding (RF), a regimen that restricts the duration of food availability with no calorie restriction, entrains the circadian clock in peripheral tissues. Restricted feeding leads to high-amplitude circadian rhythms, which have been shown to promote wellness and reduce disease and inflammatory markers. Retinoids, such as all-*trans* retinoic acid (ATRA), act as anti-inflammatory agents. Thus far, the effect of ATRA combined with RF on the ability to delay the occurrence of age-associated changes, such as cancer and inflammation, is not known. We measured circadian expression of clock genes, disease marker genes and inflammatory markers in the serum, liver and jejunum in mice fed *ad libitum* (AL) or RF supplemented with 15 or 250 μg/kg body/day ATRA for 16 weeks. Our results show that ATRA supplementation led to phase shifts and reduced amplitudes in clock genes. Under AL, ATRA reduced the average daily messenger RNA (mRNA) levels of some disease markers, such as liver *Afp* and jejunum *Afp*, *Alt* and *Gadd45*β and aspartate transaminase (AST) protein in the serum, but increased the expression level of liver *Crp* mRNA. Under RF, ATRA reduced the average daily levels of jejunum *Alt* and *Gadd45*β and AST protein in the serum, but increased liver *Afp*, *Alt*, *Gadd45*β and *Arginase* mRNA. Altogether, our findings suggest that ATRA strongly affects circadian oscillation and disease marker levels. Moreover, its impact is different depending on the feeding regimen (AL or RF).

Keywords: Circadian; Restricted feeding; Vitamin A; ATRA; Clock; Disease

1. Introduction

Circadian rhythms in mammals are regulated by the master circadian clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus [1]. A critical feature of circadian timing is the ability of the clockwork to be reset by environmental light to the 24-h day, with the retinohypothalamic tract being the principal pathway through which entrainment information reaches the SCN [2]. Synchronization among SCN neurons leads to coordinated circadian outputs from the nuclei, ultimately regulating sleep–wake cycles and rhythms in physiology and behavior [3–5]. Similar clocks are found in peripheral tissues, such as the liver and digestive system [6,7]. The clockwork consists of the transcription factor CLOCK [8] that dimerizes with BMAL1 to activate transcription by binding to enhancer E-box sequences. Thus, CLOCK and BMAL1 constitute the positive limb of the clock [1]. Periods (*Per1*, *Per2*, *Per3*) and cryptochromes (*Cry1*, *Cry2*) are induced by the CLOCK:BMAL1 heterodimer; but once the proteins are produced, they inhibit transcription and serve as the negative feedback loop of the clock [1,9,10].

High-amplitude circadian rhythms have been previously associated with aging retardation and extended lifespan. Longevity was increased in older hamsters given fetal suprachiasmatic implants that restored higher-amplitude rhythms [11,12]. Disruption of circadian rhythms by shift work or sleep deprivation or by mutations in clock genes can lead to manifestations of the metabolic syndrome, as well as certain types of cancer, coronary heart diseases, depression and overall reduced life expectancy [13–22].

Nuclear receptors constitute a large superfamily of proteins that functions as ligand-inducible transcription factors and include retinoic acid receptors (RARα/β/γ, NR1B1–3), RXRs (RXRα/β/γ, NR2B1–3), REV-ERBs and RORs [23,24]. A large number of nuclear receptors, among which are RARα and RARγ, have been found to exhibit circadian oscillation [25]. While RARs bind all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9-*cis*-RA), RXRs bind only 9-*cis*-RA [23]. Retinoids, a family of vitamin A analogs such as ATRA and 9-*cis*-RA, are used clinically for the treatment of a number of dermatologic, hematopoietic and cancerous diseases [26]. Retinoids have been demonstrated to impart significant anti-inflammatory effects in experimental trials, such as inhibition of various immune factors, including the activity of leukocytes, the release of proinflammatory cytokines and other mediators and the expression of transcription factors and toll-like receptors involved in immunomodulation [27].

In addition, retinoids affect peripheral clocks [23], as retinoic acid has been shown to up-regulate *Per1* and *Per2* expression in an E-box-dependent manner in mouse fibroblast NIH3T3 cells [28]. Similarly, retinoic acid could phase-shift *Per2* expression *in vivo* and in serum-induced smooth muscle cells *in vitro* [29]. However, when retinoic acid

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is administered to cells expressing the retinoic acid receptors RARα or RXRα, such as in vascular cells, the ligand–receptor complex competes with BMAL1 for binding to CLOCK or NPAS2, a CLOCK homolog [30,31]. These interactions negatively regulate CLOCK/NPAS2:BMAL1-mediated transcriptional activation of clock gene and clock-controlled gene expression, leading to reduced transcript levels [28,29].

Limiting the time and duration of food availability with no caloric reduction is termed restricted feeding (RF). Interestingly, diurnal RF in nocturnal animals shifts many physiological activities normally dictated by the SCN in peripheral tissues. Restricted feeding leads to robust circadian rhythms [32] and entrains rhythms of inflammation-related factors without promoting an acute-phase response [33]. It is assumed that RF affects the physiology of the animal not through the SCN but through a food-entrainable oscillator whose location has been elusive [17]. Since RF leads to high-amplitude circadian rhythms [32], which are associated with improved health [11,12], and ATRA leads to reduced inflammation [27], we investigated the effect of the combination of ATRA and RF on the expression of the circadian clock and inflammatory and disease markers.

2. Materials and methods

2.1. Animals, treatments and tissues

Nine-week-old male C57BL/6 mice were housed in a temperature- and humidity-controlled facility (23°C–24°C, 60% humidity). Mice were entrained to a light–dark cycle of 12 h light and 12 h darkness (LD) for 2 weeks with food available *ad libitum* (AL). After two weeks of AL feeding, mice were fed AL or restricted in feeding time (RF), and each group was divided into three subgroups: a control group and two groups supplemented with either 250 or 15 μg/kg/day ATRA for 16 weeks. Mice in each subgroup were housed together. The RF group was given food between ZT3 and ZT6 (ZT0 is the time of lights on). Daily food intake was measured, and body weight was monitored once weekly throughout the experiment. Average body weight and food consumption on the 8th and 16th week are shown in Table S1. After 4 months, mice were anesthetized with isoflurane and sacrificed around the circadian cycle on the first day of total darkness (DD) under dim red light to avoid the masking effects of light. Blood was drawn at two time points: CT6 and CT18 (CT0 is the time under DD conditions the lights used to turn on). These time points were selected as the midrest and midactivity points, respectively. Blood was left to clot at room temperature for 2 h, centrifuged for 10 min at 1500*g* and stored at −80°C until further analysis. Tissues were immediately frozen in liquid nitrogen and stored at −80°C until further analysis. The joint ethics committee (Institutional Animal Care and Use Committee) of the Hebrew University and Hadassah Medical Center approved this study.

2.2. Animal locomotor activity

Mice were housed individually in 17.5×28×13-cm plastic cages. After 14 days in 12:12 LD, mice were put in total darkness (DD) for 16 days. General activity was monitored using a system composed of infrared detectors (Intrusion detector model MH10; Crow group, Airport City, Israel) that were placed above each cage and connected to a computer [34]. Data were collected continuously using ADAMView software (Advantech, Milpitas, CA, USA) at 6-minute intervals.

2.3. RNA extraction and quantitative real-time polymerase chain reaction

For gene expression analyses, RNA was extracted from liver and jejunum using TRI Reagent (Sigma, Rehovot, Israel). Total RNA was DNase I-treated using RQ1 DNase (Promega, Madison, WI, USA) for 2 h at 37°C, as was previously described [6,35]. Two micrograms of DNase I-treated RNA were reverse transcribed using MMuLV reverse transcriptase (Promega) and random hexamers. One twentieth of the reaction was then subjected to quantitative real-time polymerase chain reaction (PCR) using primers spanning exon–exon boundaries and the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers for all genes were tested alongside the normalizing gene glyceraldehyde 3 phosphate dehydrogenase (*Gapdh*). The primers used are shown in Table S2. Primers were designed with Primer Express v.2 (Applied Biosystems) and validated by a standard curve and dissociation curve of the product. The fold change in target gene expression was calculated by the $2^{-\Delta\Delta Ct}$ relative quantification method (Applied Biosystems).

2.4. Western blot analysis

Liver and jejunum samples (~200 mg) were homogenized in 1-ml lysis buffer (pH 7.8, 20 mM Tris, 145 mM NaCl, 5% glycerol, 1% Triton X-100, 50 nM phenylmethylsulfonyl fluoride, 50 μM sodium fluoride, 10 μM sodium orthovanadate, 50 ng/ml aprotinin, 100 ng/ml leupeptin and 0.8 μg/ml trypsin inhibitor [Sigma]), as was described [36]. Samples were run onto a sodium dodecyl sulfate polyacrylamide gel (10% for ARGINASE and alanine aminotransferase [ALT]). After electrophoresis, proteins were semidry transferred onto nitrocellulose membranes. Blots were incubated with goat antimouse ALT polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) or goat antimouse ARGINASE (Everest Biotech, Oxfordshire, UK). Antimouse actin (MP Biomedicals, Solon, OH, USA) was used to detect actin, the loading control. Reacted membranes were washed and reacted with horseradish-peroxidase-conjugated antigoat (Santa Cruz Biotechnologies) and antimouse (Jackson ImmunoResearch, West Groove, PA, USA) IgG. The immune reaction was detected by enhanced chemiluminescence (Santa Cruz Biotechnologies). Finally, bands were quantified by densitometry and expressed as arbitrary units.

2.5. Serum protein analyses

Serum protein levels of C-reactive protein (CRP), plasminogen activator inhibitor 1 (PAI-1), interleukin-6 (IL-6) and interleukin-10 (IL-10) were determined by MILLIPLEX Multiplex-Luminex panel assay (Millipore, Billerica, MA, USA). Assays were performed according to the manufacturers' instructions.

2.6. Enzymatic colorimetric tests

Serum ALT and aspartate transaminase (AST) protein levels were determined by Cobas kits (Roche Diagnostics Limited, Burgess Hill, UK) and analyzed in Roche/Hitachi analyzer (Roche Diagnostics, Indianapolis, IN, USA). Assays were performed according to the manufacturers' instructions.

2.7. Statistical analyses

All results are expressed as means±S.E. Tukey honestly significant difference (HSD) was performed for the evaluation of significant differences in average daily expression levels of disease and inflammation genes. A one-way analysis of variance (ANOVA) (time of day) was performed to analyze circadian pattern of clock genes with several time points. For all analyses, the significance level was set at $P<.05$. Statistical analysis was performed with JMP software (version 5.1; SAS Institute Inc., Cary, NC, USA). Further analysis of circadian rhythmicity was performed using Acro software (version 3.5; Circadian Rhythm Laboratory, University of South Carolina, Walterboro, SC, USA).

3. Results

To examine the impact of ATRA in combination with timed meals on circadian rhythms, mice received food enriched with retinoic acid (15 or 250 μg/kg/day) for 16 weeks either AL or restricted to 3 h (RF). A 16-week period of time allows circadian rhythms and aging-related biomarkers to change and stabilize [37,38]. The expression levels of several clock genes and disease and inflammation markers were analyzed in the serum, liver and jejunum. We used several time points throughout the circadian cycle to measure oscillation as well as average daily levels for better assessment of total protein and messenger RNA (mRNA) levels.

3.1. Effect of ATRA on body weight and food intake

The AL and RF groups supplemented with 15 or 250 μg/kg/day ATRA gained weight throughout the experiment (Fig. 1A). Nevertheless, AL feeding supplemented with 250 μg/kg/day ATRA and all the RF groups showed reduction in the final mean body weight (Fig. 1B). To test the reduction in body weight, we corrected food consumption to body mass on the 8th and 16th week (Table S1). At the end of the experiment, the average food intake to total body mass ratio of all groups ranged between 93% and 102% compared to the AL group (Table S1). As weight was gained throughout the experiment and calorie restriction is defined as 60%–75% food intake of AL [39], these results indicate that the RF-fed mice were not calorically restricted.

3.2. Effect of ATRA on locomotor activity

ATRA supplementation did not affect the pattern of circadian locomotor activity (Fig. 1C) or the 24-h activity of AL-fed mice held under LD conditions (Fig. 1D). Expectedly, the RF groups exhibited food anticipatory activity during the light period and increased activity during feeding time (Fig. 1C). Nevertheless, their

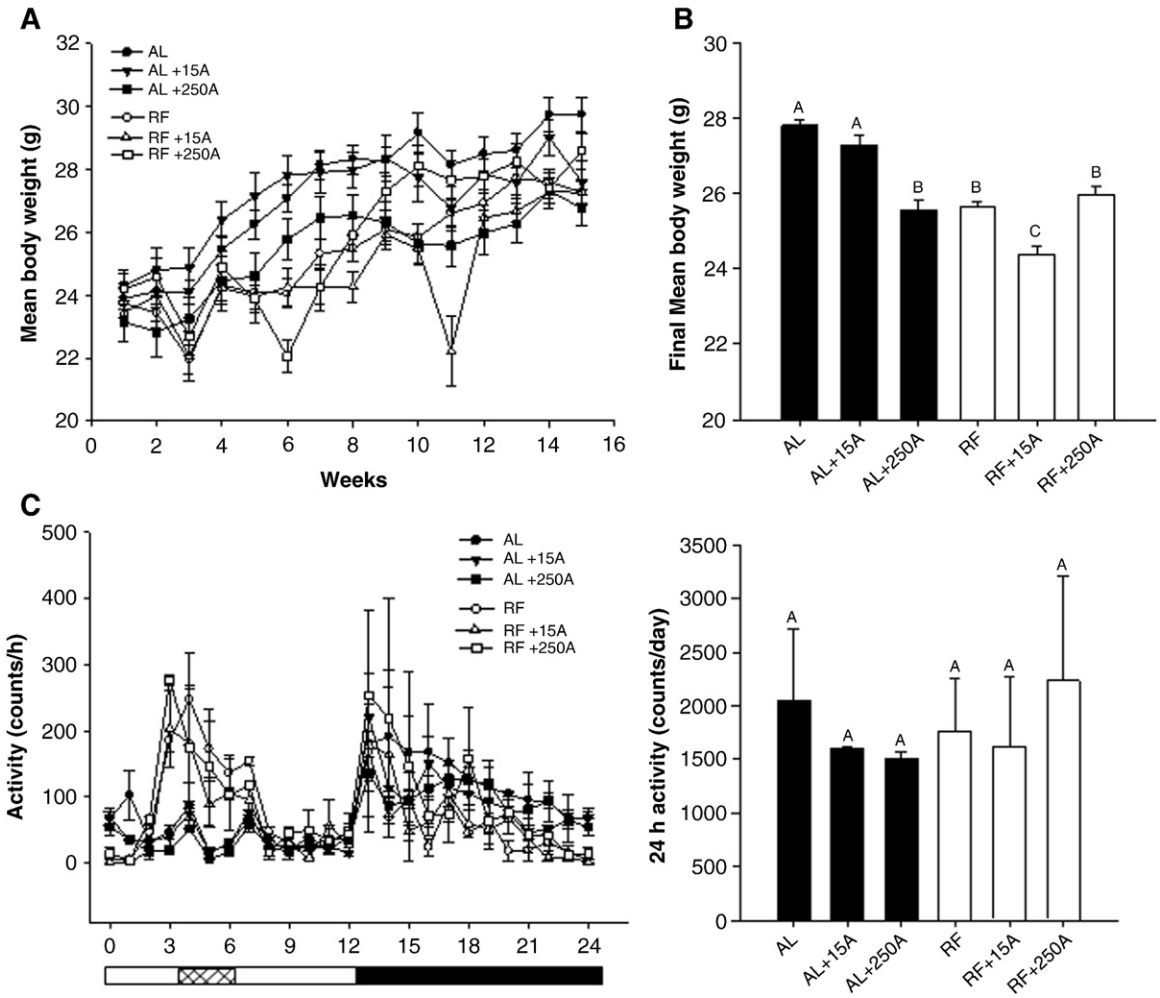


Fig. 1. Body weight and locomotor activity with or without ATRA supplementation. (A) Weekly mean body weight throughout the experiment. (B) Final mean body weight after 16 weeks. (C) Activity (number of counts per hour). (D) Twenty-four-hour activity in LD (counts per day). Mice were fed either AL or RF with no supplementation or with supplementation with 15 μg/kg body/day ATRA (15A) or 250μg/kg body/day ATRA (250A). The white and black bars designate the day and night, respectively. Food availability during RF is marked by the crosshatched box. Values are mean±S.E.; $n=6$ for each group. Different letters denote significant differences ($P<.05$) by Tukey HSD.

total 24-h activity was not different than the total 24-h activity of the AL groups (Fig. 1D). ATRA did not affect locomotor activity under AL or RF even under dark–dark conditions (DD) (data not shown).

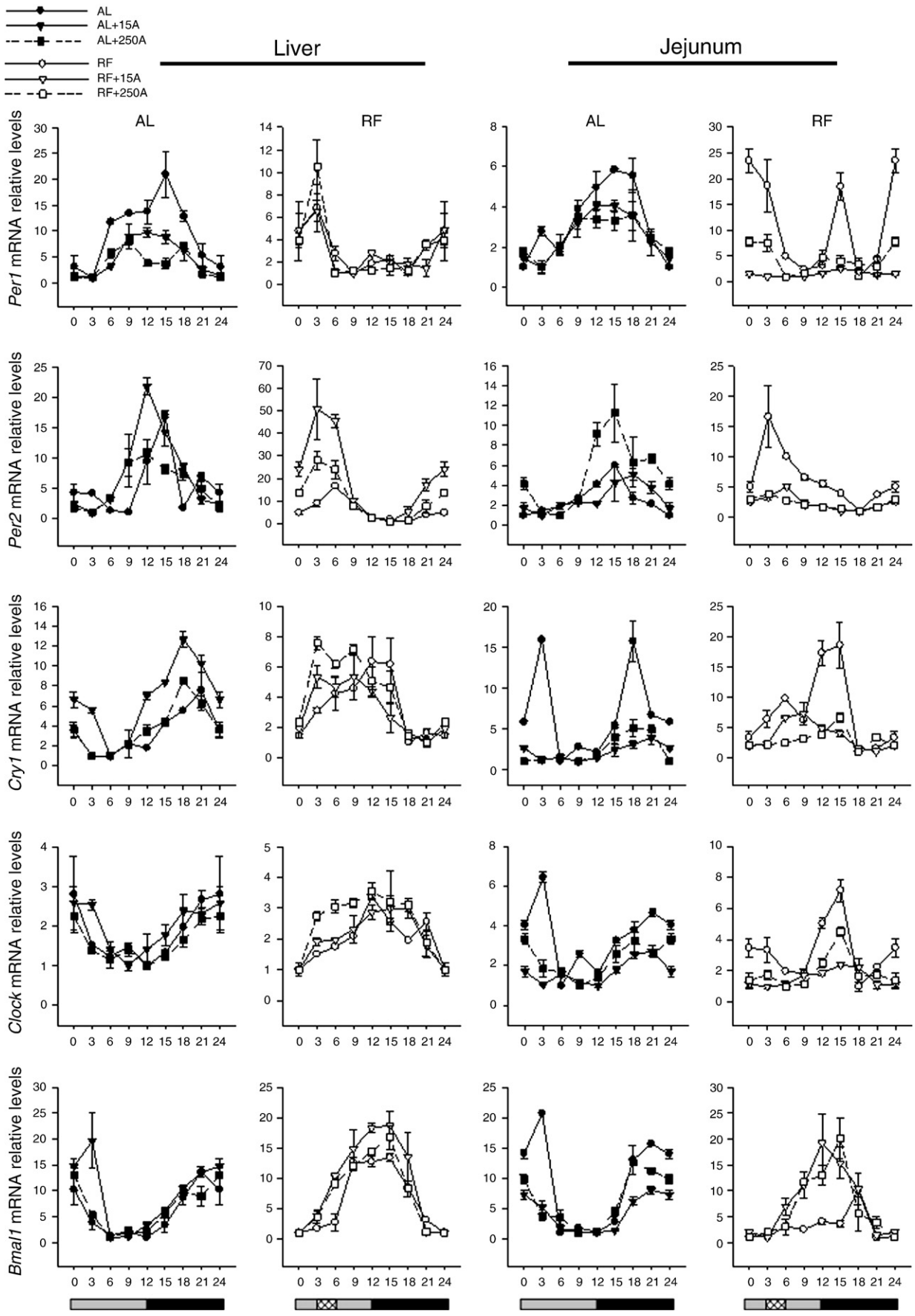
3.3. Effect of ATRA on clock gene expression

To study the circadian clock of mice under AL and RF supplemented with ATRA, we tested the phase and amplitude of the clock genes *Per1*, *Per2*, *Cry1*, *Clock* and *Bmal1* at the mRNA level in mouse liver and jejunum. Our analyses revealed that all clock genes oscillated robustly in all groups ($P<.05$, one-way ANOVA), except for *Per1* in the jejunum under RF supplemented with 15 μg/kg/day (Fig. 2, Table S3). All RF groups exhibited a phase shift in the expression of clock genes compared with the AL groups (Fig. 2, Table S4). In the liver, ATRA supplementation resulted in a phase advance mainly of *Per2* and *Cry1* mRNA under AL and of *Cry1* and *Bmal1* mRNA under RF (Fig. 2, Table S4). In the jejunum, ATRA supplementation resulted in a phase shift mainly of *Clock*, *Bmal1* and *Per2* under AL (Fig. 2, Table S4). The 15-μg/kg/day ATRA supplementation under AL resulted in reduced amplitudes of liver *Per1* and *Clock* mRNA and *Per1*, *Cry1* and *Clock* mRNA under RF (Fig. 2, Table S5). The 250-μg/kg/day ATRA supplementation led to reduced amplitudes of liver *Clock*, *Bmal1*, *Per1* and *Per2* under AL and *Per1* mRNA under RF (Fig. 2, Table S5). In the jejunum, we found reduction in the amplitudes of all clock genes under AL with 15 μg/kg/day ATRA (Fig. 2, Table S5). Supplementation with 250 μg/kg/day ATRA under AL led to reduction in *Per1* and *Cry1* mRNA amplitudes (Fig. 2, Table S5). In the jejunum, we also found a reduction in all clock genes except for *Bmal1* mRNA under RF supplemented with 15 μg/kg/day ATRA (Fig. 2, Table S5). Supplementation with 250 μg/kg/day ATRA under RF led to reduction in *Per2* and *Cry1* mRNA amplitudes (Fig. 2, Table S5). Our results indicate that both phase shifts and amplitude reduction were seen in clock gene expression with ATRA supplementation.

3.4. Effect of ATRA on the levels of disease markers

We next studied the effect of ATRA supplementation combined with AL and RF on age-related diseases by assessing several disease markers for their phase, amplitude and overall expression levels around the circadian cycle. All results with ATRA supplementation were compared to their corresponding feeding: AL or RF (Table S6).

We first measured alpha fetoprotein (AFP), a marker for hepatocellular carcinoma [40]. The AL diet supplemented with 15 μg/kg/day ATRA significantly down-regulated *Afp* mRNA daily levels in the liver to 42% (Fig. 3, Table S6). Restricted feeding did not affect *Afp* mRNA levels, but supplementation with 15 μg/kg/day ATRA


AL
AL+15A
AL+250A
RF
RF+15A
RF+250A
Liver
Jejunum
AL
RF
AL
RF
Per1 mRNA relative levels
Per2 mRNA relative levels
Cry1 mRNA relative levels
Clock mRNA relative levels
Bmal1 mRNA relative levels
0 3 6 9 12 15 18 21 24

significantly up-regulated *Afp* mRNA daily levels to 224% compared to AL feeding (Fig. 3, Table S6). In the jejunum, AL feeding supplemented with 15 μg/kg/day ATRA significantly down-regulated *Afp* mRNA daily levels to 48% (Fig. 3, Table S6). The RF diet significantly down-regulated *Afp* mRNA daily levels to 49%, and supplementation with 250 μg/kg/day ATRA increased the levels back to AL group levels (Fig. 3, Table S6).

Next we measured ALT, a marker for nonalcoholic fatty liver and obesity [41]. Average daily levels of liver *Alt* mRNA did not significantly change under AL diet supplemented with ATRA (Fig. 3, Table S6). Liver average daily levels of ALT protein (Fig. 4) and serum ALT protein (Fig. 5) levels also were not significantly affected by ATRA. Restricted feeding up-regulated *Alt* mRNA levels up to 140%, but ATRA supplementation did not affect *Alt* mRNA levels, indicating that ATRA by itself did not have any effect (Fig. 3, Table S6). In the jejunum, 250 μg/kg/day ATRA supplementation under AL or RF significantly decreased *Alt* mRNA daily levels to 59% and 63%, respectively (Fig. 3, Table S6).

Growth arrest and DNA damage 45β (GADD45β), a marker for hepatocellular carcinoma [42], was then measured. In the liver, AL feeding supplemented with ATRA did not significantly change *Gadd45*β mRNA average daily levels (Fig. 3, Table S6). The RF diet did not affect *Gadd45*β mRNA levels, but supplementation with 250 μg/kg/day ATRA significantly up-regulated *Gadd45*β mRNA levels to 232% (Fig. 3, Table S6). In the jejunum, AL feeding supplemented with 250 μg/kg/day ATRA significantly down-regulated *Gadd45*β mRNA levels to 66% (Fig. 3, Table S6). Daily average levels of *Gadd45*β under RF were significantly lower than those under AL ($P<.05$, Student's test; $P>.05$, Tukey HSD). Restricted feeding supplemented with 15 μg/kg/day ATRA down-regulated *Gadd45*β mRNA levels to 58% (Fig. 3, Table S6).

We next measured PAI-1, a marker for thrombosis and proneness to heart attacks [43], as it is expressed in mouse liver and gut [44,45]. Liver and jejunum *Pai-1* mRNA daily levels under AL and RF with ATRA supplementation did not significantly change (Fig. 3, Table S6). However, jejunum *Pai-1* mRNA daily levels under RF were significantly higher than those under AL ($P<.05$, Student's *t* test; $P>.05$, Tukey HSD). No significant change was detected in serum PAI-1 protein levels (Fig. 5).

We next measured *Arginase*, a marker for colorectal cancer and liver metastases [46–50]. The AL feeding with ATRA supplementation did not significantly change average daily expression levels of *Arginase* mRNA in the liver (Fig. 3, Table S6). The RF diet supplemented with 250 μg/kg/day ATRA significantly up-regulated *Arginase* mRNA daily levels to 126% (Fig. 3, Table S6). Average daily levels of liver ARGINASE protein daily levels were not affected by ATRA (Fig. 4). In the jejunum, AL and RF feeding with ATRA supplementation did not significantly change expression of daily *Arginase* mRNA levels (Fig. 5, Table S6). However, daily average levels of *Arginase* mRNA under RF were significantly lower than those under AL ($P<.05$, Student's *t* test; $P>.05$, Tukey HSD).

Aspartate transaminase, a marker for hepatotoxicity [51], was then measured. Serum AST protein daily average levels were significantly decreased under AL feeding supplemented with 15 μg/kg/day ATRA (Fig. 5). The 250-μg/kg/day ATRA supplementation increased the levels back to AL group levels. The RF diet did not affect AST protein daily average levels, but RF supplementation with 15 or 250 μg/kg/day ATRA significantly decreased AST protein levels (Fig. 5).

Most disease markers tested exhibited no robust oscillation at the mRNA level under Acro analysis. However, 15 μg/kg/day ATRA led to the oscillation of liver *Alt* mRNA under AL and jejunum *Alt*, *Gadd45*β and *Pai-1* mRNA under AL or RF, and 250 μg/kg/day ATRA led to the oscillation of liver *Gadd45*β mRNA under RF (Fig. S1, Table S7).

3.5. Effect of ATRA on inflammation markers

As ATRA supplementation is considered beneficial in a number of inflammatory conditions [52], we examined its capacity to ameliorate the expression of several inflammation markers under AL and RF. We assessed the levels of several well-established inflammatory markers [53–55] such as CRP, a marker for inflammation and coronary heart disease [56]; IL-6; and the anti-inflammatory cytokine IL-10. *Crp* mRNA daily average levels were significantly up-regulated to 147% in the liver under AL diet supplemented with 250 μg/kg/day ATRA, but with no change in the jejunum (Fig. 3, Table S6) or serum protein levels (Fig. 5). However, daily average levels of liver *Crp* mRNA under RF were significantly higher than those under AL ($P<.05$, Student's *t* test; $P>.05$, Tukey HSD) (Table S6). Serum protein levels of both IL-6 and IL-10 were not significantly different under AL and RF supplemented with 15 or 250 μg/kg/day ATRA (Fig. 5).

4. Discussion

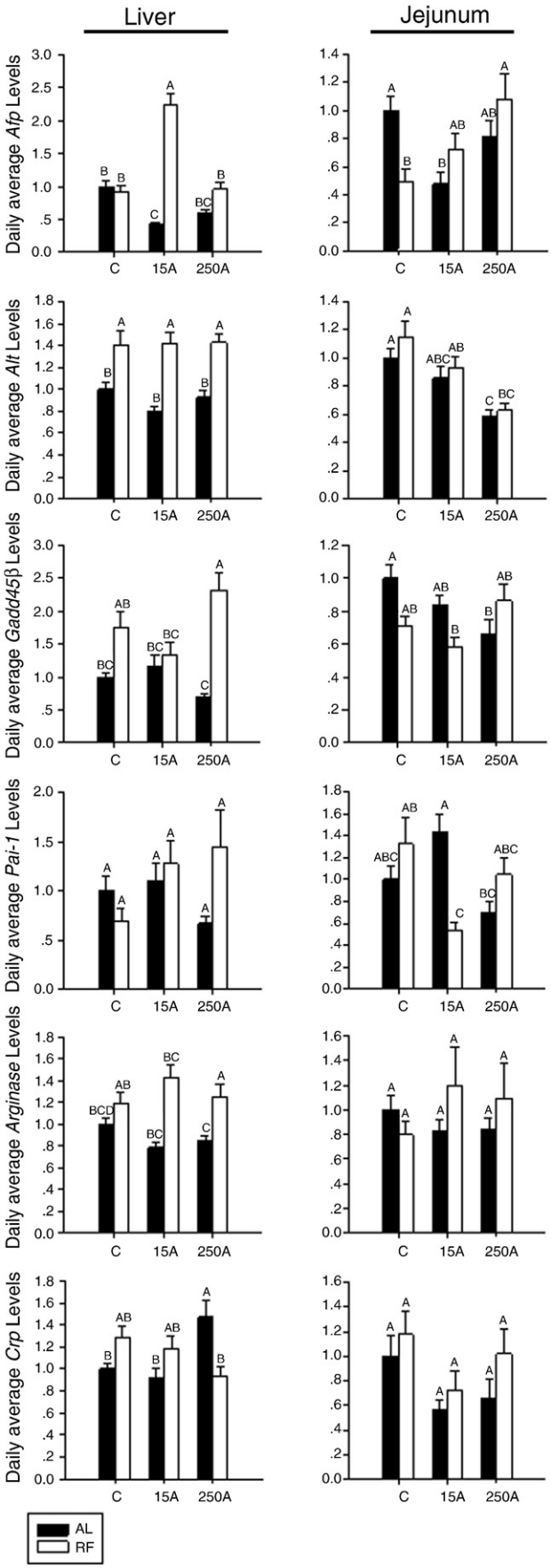
In this study, we investigated the effect of long-term ATRA treatment under AL and RF regimens on several physiological and molecular parameters under the influence of the circadian clock. ATRA did not affect locomotor activity, but we found major effects on circadian oscillation and disease marker levels.

4.1. Effect of ATRA on circadian rhythms

Our data show that clock genes are expressed and exhibit circadian oscillations in the liver and jejunum under both AL and RF combined with ATRA. This is consistent with circadian expression of clock genes and other key proteins involved in macronutrient absorption in the intestine [57]. Mice fed AL supplemented with ATRA exhibit generally lower amplitudes in the circadian expression of clock genes in the liver and jejunum (Fig. 2). Amplitude reduction can be explained by the fact that retinoid receptors (RAR, RXR) can affect expression by competing with BMAL1 for CLOCK binding, which leads to the inhibition of CLOCK:BMAL1-mediated transcriptional activation [29]. The reduced amplitudes are presumably due to the induced expression of ATRA receptors after 4 months of the treatment. Our findings are in agreement with ATRA treatment in NIH3T3 cells, which led to a dose-dependent inhibition in E-box-dependent reporter activity in the presence of exogenous RAR [28]. Under RF, less amplitude reduction was found than AL, presumably as a result of weaker inhibition of CLOCK:BMAL1-mediated transcriptional activation. This could stem from the fact that mice were exposed to ATRA only for 3 h, and the ATRA receptors could have been saturated and reached their maximal activity only transiently. In addition, the changes were less pronounced under RF regimen as this is a dominant treatment that dictates rhythms in peripheral tissues [58–63].

Our results also showed that ATRA supplementation led to phase shifts. Indeed, retinoic acid injected intraperitoneally induced phase shifts in *Per2* mRNA rhythmicity in the aorta and heart *in vivo* and in the *hPer2* rhythm in serum-shocked smooth muscle cells [29]. The lower amplitudes of *Clock* and *Bmal1* expression achieved by ATRA supplementation (Fig. 2, Table S5) could lead to phase shifts and

Fig. 2. Circadian mRNA expression of clock genes in the liver and jejunum with or without ATRA supplementation. Tissues were collected every 3 h around the circadian cycle from mice fed either AL or RF with no supplementation or with supplementation with 15 μg/kg body/day ATRA (15A) or 250 μg/kg body/day ATRA (250A). Total RNA was extracted, and real-time PCR analyses were performed to determine mRNA levels of *Per1*, *Per2*, *Cry1*, *Clock* and *Bmal1*. The gray and black bars designate the subjective day and night, respectively. Food availability during RF is marked by the crosshatched box. Values are mean±S.E.; $n=4$ for each time point in each group.



altered amplitudes in the expression of other clock genes. Such changes could also explain circadian oscillation seen with some disease markers after ATRA supplementation under AL or RF (Fig. S1).

4.2. ATRA leads to modified expression of disease and inflammatory markers

Retinoid receptors (RAR, RXR) regulate expression of target genes by direct binding to retinoic acid response elements. Alternatively, they can affect expression by competing with BMAL1 for CLOCK binding which inhibits CLOCK:BMAL1-mediated transcriptional activation [29]. This inhibition could lead to hampered expression of other transcription factors, such as activator protein-1 (AP-1) [64], nuclear factor-κB (NFκB) [65] and cAMP-responsive element binding protein [66]. This reduction could lead to a subsequent reduction in the expression of disease markers. Our results showed unaltered levels of the acute phase proteins in the serum IL-6 and CRP with ATRA supplementation (Fig. 5). Other reports that demonstrated a preventive effect on inflammatory state in acne patients by reducing the production of IL-6 used 13-*cis*-retinoic acid rather than ATRA [67,68]. However, measurement of serum aminotransferases revealed reduced levels of AST with ATRA (Fig. 5) as was reported [67]. No significant difference in the average values of ALT was observed (Fig. 5), similarly to what was reported during treatment with 13-*cis*-retinoic acid [68]. Moreover, acyclic retinoid administration did not decrease serum ALT activity in patients who were enrolled in the clinical trial to prevent second primary hepatocellular carcinoma [69].

ATRA-treated mice exhibited low daily expression levels of *Afp* mRNA under AL in the liver and jejunum (Fig. 3). Indeed, recent studies show that induction of both differentiation and apoptosis by acyclic retinoids in hepatocellular carcinoma cells down-regulated AFP expression, a marker of immature, latent or transformed hepatocytes [69–71]. ATRA also decreased jejunal *Gadd45*β mRNA daily expression levels under AL (Fig. 3, Table S6). This decrease may be linked to the reduction of stress-related immune factors, such as tumor necrosis factor α (TNFα) [72]. On the other hand, ATRA increased liver *Gadd45*β mRNA daily levels under RF (Fig. 3, Table S6). Since *Gadd45*β mRNA reduction is detrimental and may lead to hepatocellular DNA damage [42], our observations suggest a beneficial outcome.

Under RF feeding combined with ATRA, mice exhibited low daily expression levels of jejunum *Alt* and *Gadd45*β mRNA (Fig. 3, Table S6) and reduced levels of AST protein (Fig. 5). Recently, we have found that RF by itself led to the reduction of liver *Il-6* mRNA, TNFα and NFκB protein; jejunum *Arginase*, *Afp*, *Gadd45*β, *Il-1*α and *Il-1*β mRNA; and IL-6 and TNFα protein [73]. In contrast, the anti-inflammatory cytokine IL-10 mRNA increased in the liver and jejunum [73]. Herein we show that several disease markers in the liver showed increased expression under RF feeding combined with ATRA, such as liver *Afp*, *Gadd45*β and *Arginase*. This discrepancy can be explained by the beneficial effects of high-amplitude rhythms achieved by RF [11,73] but the low-amplitude rhythms caused by ATRA. The RF regimen resembles the month of Ramadan, during which Muslims abstain from eating and drinking during day hours from dawn to sunset and fast for about 16 h a day, while the last meal is taken at 1:00–2:00 AM [74]. In light of our results, it seems that

Fig. 3. Daily average mRNA expression of disease and inflammation markers in the liver and jejunum with or without ATRA supplementation. Tissues were collected every 3 h around the circadian cycle from mice fed either AL or RF with no supplementation (C, control group) or with supplementation with 15 μg/kg body/day ATRA (15A) or 250 μg/kg body/day ATRA (250A). Total RNA was extracted, and real-time PCR analyses were performed to determine mRNA levels of *Afp*, *Alt*, *Gadd45*β, *Pai-1*, *Arginase* and *Crp*. Data from all time points were combined and averaged. Values are mean±S.E.; $n=16$ for each group. Different letters denote significant differences ($P<.05$) by Tukey HSD.

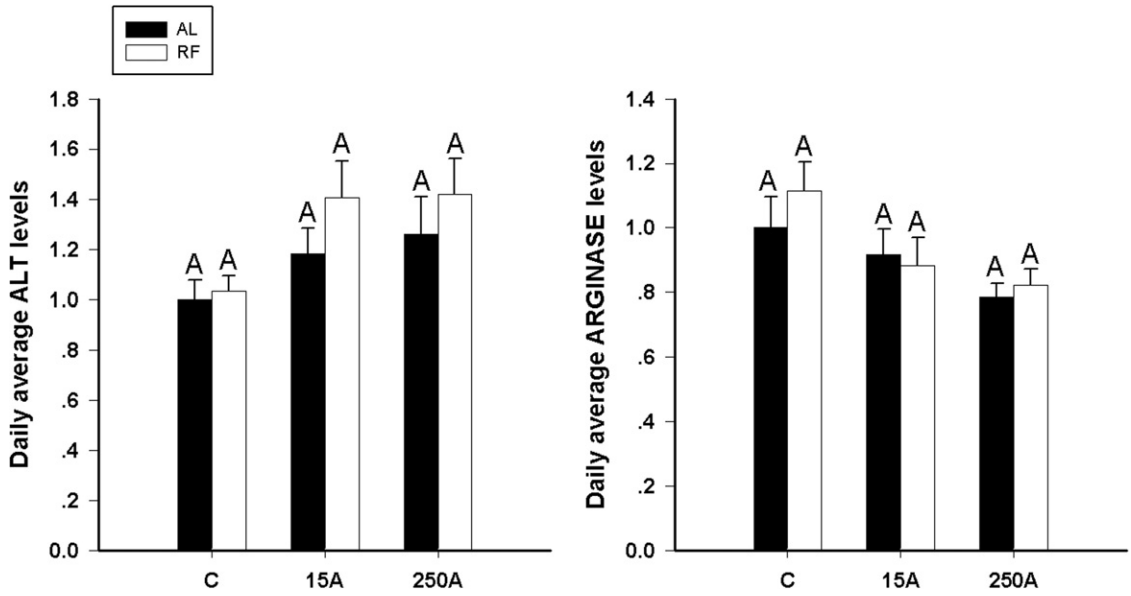


Fig. 4. ALT and ARGINASE daily average protein levels in the liver with or without ATRA supplementation. Tissues were collected every 3 h around the circadian cycle from mice fed either AL or RF with no supplementation (C, control group) or with supplementation with 15 μg/kg body/day ATRA (15A) or 250 μg/kg body/day ATRA (250A). Protein was quantified by Western blotting. Data from all time points were combined and averaged. Values are mean±S.E.; $n=16$ for each group. Different letters denote significant differences ($P<.05$).

ATRA supplementation should not be recommended during the month of Ramadan.

Altogether, our results show the effect of ATRA on circadian oscillation leading to shifted and low-amplitude rhythms. Reduction in disease marker expression levels was mainly seen in the jejunum when ATRA supplementation was combined with AL. Although RF leads to robust circadian rhythms [32] and entrains rhythms of inflammation-related factors [33], ATRA supplementation counteracts these beneficial effects by shifting and lowering the levels of gene expression mainly in the liver.

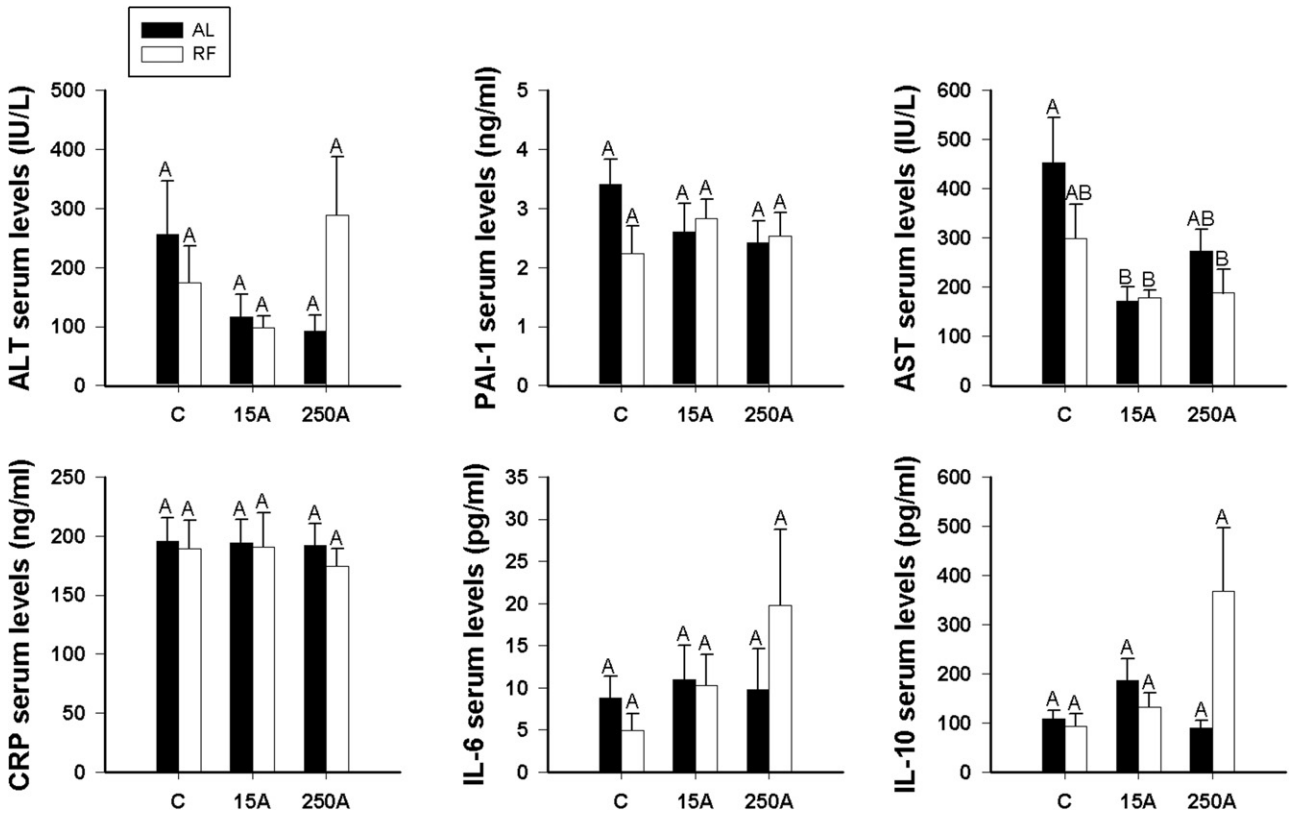


Fig. 5. Disease and inflammation marker serum levels with or without ATRA supplementation. Blood was collected, and serum was separated for analyses under DD at the subjective midday and midnight from mice fed either AL or RF with no supplementation (C, control group) or with supplementation with 15 μg/kg body/day ATRA (15A) or 250 μg/kg body/day ATRA (250A). Protein levels were determined by Multiplex-Luminex panel assay for CRP, PAI-1, IL-6 and IL-10 and by Cobas kits for ALT and AST. Data were averaged from these time points. Values are mean±S.E.; $n=6$ for each group. Different letters denote significant differences ($P<.05$) by Tukey HSD.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.11.017.

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