

# Liver Injury in Acute Fatty Liver of Pregnancy: Possible Link to Placental Mitochondrial Dysfunction and Oxidative Stress

Sathish Kumar Natarajan,<sup>1</sup> Kavitha R. Thangaraj,<sup>1</sup> C. E. Eapen,<sup>1</sup> Anup Ramachandran,<sup>1</sup> Ashis Mukhopadhyaya,<sup>1</sup> Mathews Mathai,<sup>2</sup> Lakshmi Seshadri,<sup>2</sup> Abraham Peedikayil,<sup>2</sup> Banumathi Ramakrishna,<sup>3</sup> and Kunnissery A. Balasubramanian<sup>1\*</sup>

Acute fatty liver of pregnancy (AFLP) is a rare disorder which is fatal if not recognized and treated early. Delivery of the fetoplacental unit results in dramatic improvement in maternal liver function, suggesting a role for the placenta. However, the mechanisms by which defects in the fetus or placenta lead to maternal liver damage are not well understood and form the focus of this study. Placenta and serum were obtained at delivery from patients with AFLP, and placental mitochondria and peroxisomes were isolated. Placental mitochondrial function, oxidative stress, and fatty acid composition as well as serum antioxidants, oxidative and nitrosative stress markers, and fatty acid analysis were carried out. Hepatocytes in culture were used to evaluate cell death, mitochondrial function, and lipid accumulation on exposure to fatty acids. Oxidative stress was evident in placental mitochondria and peroxisomes of patients with AFLP, accompanied by compromised mitochondrial function. Increased levels of arachidonic acid were also seen in AFLP placenta when compared to control. Patients with AFLP also had a significant increase in oxidative and nitrosative stress markers in serum, along with decreased antioxidant levels and elevated levels of arachidonic acid. These levels of arachidonic acid were capable of inducing oxidative stress in hepatocyte mitochondria accompanied by induction of apoptosis. Exposure to arachidonic acid also resulted in increased lipid deposition in hepatocytes. **Conclusion:** Oxidative stress in placental mitochondria and peroxisomes is accompanied by accumulation of toxic mediators such as arachidonic acid, which may play a causative role in maternal liver damage seen in AFLP. (HEPATOLOGY 2010;51:191–200.)

Acute fatty liver of pregnancy (AFLP) is an example of a primary mitochondrial hepatopathy<sup>1</sup> characterized by hepatic microvesicular steatosis, hepatic failure, and encephalopathy developing in the last trimester

of pregnancy.<sup>2,3</sup> Although the majority of primary mitochondrial hepatopathies present in childhood, AFLP presents in a previously asymptomatic woman in late pregnancy. The disease is associated with defects in  $\beta$ -oxidation of fatty acids in mitochondria<sup>4</sup> especially the mitochondrial long-chain acyl coenzyme A dehydrogenase (LCHAD)<sup>5,6</sup> in the fetus, but it is now recognized that AFLP can occur without a mutation in LCHAD.<sup>7,8</sup> This suggests that the metabolic basis of AFLP is more heterogeneous than believed earlier, but the mechanism by which a fetal defect in lipid metabolism causes maternal liver damage is not well understood. Interestingly, it has been observed that patients with AFLP generally recover from liver dysfunction subsequent to delivery of the fetus,<sup>9</sup> suggesting a causative role for the placenta, which is expelled during delivery.

During gestation, the placenta is essential for fetal development and utilizes fatty acids as a significant metabolic fuel.<sup>10</sup> The genetic composition of the placenta is identical to that of the fetus, and all enzymes of the mitochondrial fatty acid  $\beta$ -oxidation pathway are expressed and active in human placenta,<sup>11</sup> with activities being maximum in the second trimester and decreasing with gesta-

AFLP, acute fatty liver of pregnancy; EDTA, ethylene diamine tetraacetic acid; LCHAD, long-chain acyl coenzyme A dehydrogenase; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide.

From the <sup>1</sup>The Wellcome Trust Research Laboratory, Department of Gastrointestinal Sciences, <sup>2</sup>Department of Obstetrics and Gynecology, <sup>3</sup>Department of Pathology, Christian Medical College, Vellore, India.

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\*Present address: Department of Biochemistry, Meenakshi Medical College and Research Institute, Enathur, Kanchipuram-631552, Tamilnadu, India.

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Address reprint requests to: K. A. Balasubramanian, Ph.D., or Anup Ramachandran, Ph.D., The Wellcome Trust Research Laboratory, Department of Gastrointestinal Sciences, Christian Medical College, Ida Scudder Road, Vellore-632004, India. E-mail: (K.A.B.) wlbalu@hotmail.com; wellcome@cmcvellore.ac.in; or (A.R.) anup@cmcvellore.ac.in; wellcome@cmcvellore.ac.in; fax: (91) (0416) 2282486.

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tional age in the third trimester.<sup>4</sup> It is also recognized that placental fatty acid metabolism can play a critical role in guiding pregnancy and fetal outcome.<sup>12</sup> Defects in transport of fatty acids into the mitochondria or blocks at any other steps of  $\beta$ -oxidation could lead to accumulation of fatty acids and their metabolic products,<sup>13</sup> which could be toxic.

Oxidative stress has been implicated in a number of liver diseases, and earlier work from our laboratory demonstrated that experimental hepatic microvesicular steatosis results in mitochondrial dysfunction and oxidative stress in liver subcellular organelles.<sup>14</sup> Oxidative stress has been demonstrated in patients with preeclampsia,<sup>15</sup> a condition seen in 40% of patients with AFLP.<sup>16</sup> Mitochondria are an important cellular source of free radicals, and placental mitochondria have been implicated in free radical generation in patients with preeclampsia.<sup>17</sup> Another cellular source of free radicals in relation to fatty acid oxidation would be peroxisomes, where  $\beta$ -oxidation of fatty acids can result in generation of these active species.<sup>18</sup>

Based on the dramatic improvement in maternal liver function on delivery and the fact that the mother preferentially uses fatty acids as the predominant energy source in late pregnancy,<sup>19</sup> we hypothesized that compromised fatty acid metabolism in placental mitochondria of patients with AFLP would result in generation of oxidative stress in mitochondria and peroxisomes with production of toxic intermediates of fatty acid oxidation. These compounds could then spill over into the maternal circulation and result in hepatotoxicity. This hypothesis was tested by evaluation of oxidative stress in serum as well as analysis of placental mitochondria and peroxisomes from patients with AFLP in comparison with controls.

## Patients and Methods

Adenosine diphosphate; dimethyl sulfoxide; 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); 1,1', 3,3'-tetramethoxy propane, Tris(hydroxymethyl) aminomethane (Tris); thiobarbituric acid; dithio-bis-(2-nitrobenzoic acid); 2,4-dinitrophenyl hydrazine; arsenazo III; succinic acid; 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium; bovine serum albumin; arachidonic acid; caspase-3 substrate (AC-DEVD-pNA); dithiothreitol; dihydrorhodamine (DHR); Nile red; and *para*-nitrophenyl phosphate were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents used were of analytical grade.

**Patients.** Seven patients with AFLP diagnosed as per clinical diagnostic criteria<sup>20</sup> were recruited for the study, after obtaining informed consent. Five of these patients underwent liver biopsy and had hepatic microvesicular

steatosis (Fig. 1), which confirmed the diagnosis of AFLP in these patients. All seven patients had negative hepatitis A, B, C, and E serology. None of the AFLP patients were obese. Controls were 10 mothers matched for gestational age, who underwent normal delivery at the hospital. Both controls and AFLP patients were from the lower socioeconomic category. This study was approved by the Institutional Experimentation Ethics Committee.

**Isolation of Subcellular Organelles.** Human placentas were processed within 30 minutes of delivery. The blood was drained and placental tissue was minced and washed three times with a buffer containing 250 mM sucrose, 1 mM Tris, and 1 mM ethylene diamine tetraacetic acid (EDTA; pH 7.4). The washed mince was filtered through two layers of surgical gauze and homogenized with four volumes of buffer containing 250 mM sucrose, 1 mM Tris, 1 mM EDTA, pH 7.4. The homogenate was centrifuged at 1500g for 15 minutes and mitochondria were pelleted at 16,000g for 10 minutes and washed twice with mitochondrial suspension buffer containing 250 mM sucrose, 1 mM Tris HCl (pH 7.4).<sup>21</sup> Postmitochondrial supernatant was then centrifuged at 39,000g for 10 minutes to isolate the fraction including peroxisomes, which was resuspended in 250 mM sucrose containing 1 mM EDTA and 1 mM Tris HCl (pH 7.4). This suspension was again centrifuged at 16,000g for 10 minutes to remove mitochondrial contamination and the supernatant was then centrifuged at 39,000g for obtaining peroxisomal fraction. Purity of the isolated mitochondria and peroxisomes was checked by enrichment of marker enzymes succinate dehydrogenase and catalase,<sup>14</sup> respectively.

**Assessment of Placental Mitochondrial Function.** Mitochondrial function was assessed by measuring oxygen uptake, mitochondrial swelling, MTT reduction, and calcium flux measurements. Oxygen uptake was determined polarographically using a Clark-type electrode in 3 mL respiration medium (150 mM sucrose, 1 mM  $\text{KH}_2\text{PO}_4$ , 10 mM Tris, 5 mM  $\text{MgCl}_2$ , 20 mM KCl [pH 7.4]) containing 5 mM succinate as respiratory substrate. A mitochondrial protein of 1-2 mg/mL was used. Oxygen uptake during both state 3 (in presence of adenosine diphosphate and succinate) and state 4 (in presence of succinate alone) respiration were measured and the ratio of state 3/state 4 respiratory rate was used to calculate the respiratory control ratio.<sup>22</sup> A mitochondrial protein corresponding to 100-200  $\mu\text{g}$  in suspension buffer was used to determine mitochondrial swelling by measuring the decrease in absorbance at 540 nm up to 7 minutes. To determine the exclusive effect of AFLP on placental mitochondrial swelling without additional stimuli, no external inducers such as calcium or oxidative stress were

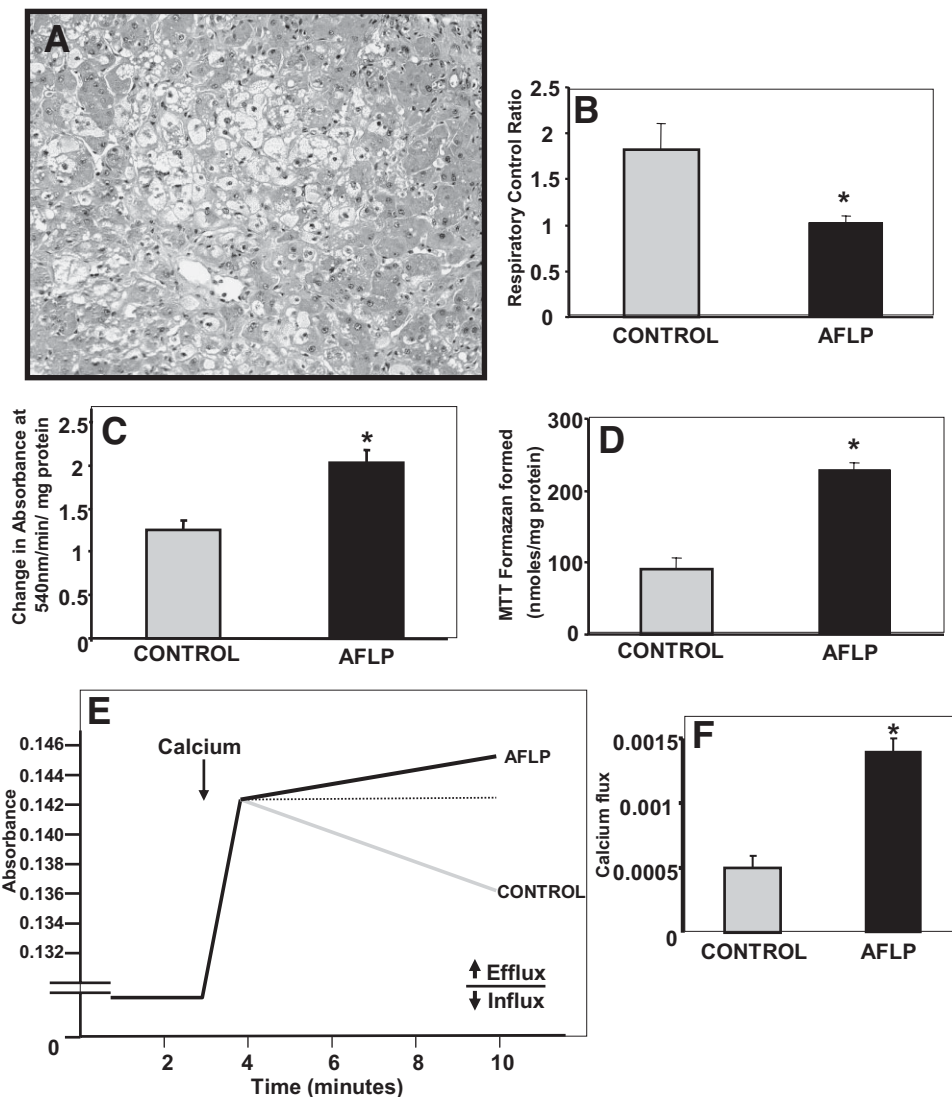


Fig. 1. (A) Liver biopsy showing microvesicular steatosis in the perivascular region. (B-E) Mitochondrial function in placenta. (B) Respiratory control ratio, (C) mitochondrial swelling, (D) MTT reduction, (E) a representative trace from calcium flux measurements, and (F) quantitative assessment of calcium efflux in placental mitochondria isolated from patients with AFLP ( $n = 7$ ), when compared with healthy pregnant controls ( $n = 10$ ). The assays were done as described in the text. Each value represents mean  $\pm$  SD. \*  $P < 0.05$ , when compared to control.

applied in these experiments. The data is expressed as change in absorbance per minute per mg protein.<sup>23</sup> The MTT reduction assay was performed using a microplate reader as described.<sup>24</sup> Calcium flux measurement was done by quantitation of changes in the absorption spectrum of Arsenazo III at 675/685 nm. Arsenazo III is an impermeable dye that indicates calcium concentration outside mitochondria. On addition of exogenous calcium to a suspension of mitochondria, there is an initial increase in absorbance due to binding of calcium to the dye. However, this is a dynamic process, and as the mitochondria take up calcium through the uniporter, there is a decrease in external concentrations of calcium, reflected in a decrease in absorbance. Mitochondria were suspended in a medium containing 230 mM mannitol, 70 mM sucrose, 5 mM HEPES, 5 mM succinate, and 40  $\mu$ M Arsenazo III (pH 7.4). A concentration of 10  $\mu$ M calcium was added to the reaction medium to initiate the flux studies.<sup>25</sup>

**Measurement of Oxidative Stress Markers.** Malondialdehyde was measured using the thiobarbituric acid method.<sup>26</sup> For conjugated diene measurements, total lipids were extracted, dissolved in 1 mL heptane, read at 233 nm and expressed as nmoles per milligram protein using a molar absorption coefficient of  $2.52 \times 10^4$ . Protein carbonyl content formed was measured using 2,4-dinitrophenyl hydrazine and calculated using an extinction coefficient of  $22 \text{ mM}^{-1} \text{ cm}^{-1}$ .<sup>27</sup> Protein thiol and total thiol content were measured using dithio-bis-(2-nitrobenzoic acid) and expressed as nanomoles per milligram protein.<sup>28</sup> Protein was estimated by Lowry's method using bovine serum albumin as a standard.<sup>29</sup>

**Enzyme Assays.** Catalase activity was estimated by measuring the change in absorbance at 240 nm using hydrogen peroxide as substrate and expressed as units per milligram protein<sup>30</sup> (units are expressed as micromoles per minute). Succinate dehydrogenase activity was assayed using 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phe-

nyl tetrazolium as an electron acceptor, which forms formazan crystals on reduction. Data is expressed as units per milligram protein.<sup>31</sup>

**Serum Retinol and Tocopherol Extraction and Quantitation by High-Performance Liquid Chromatography.** For analysis, 0.5 mL serum was mixed with equal volume of ethanol and hexane. Retinol and tocopherol were extracted three times by a triple volume of hexane. Extracted fractions were dried under nitrogen and reconstituted in methanol:diethyl ether (3:1), followed by separation and quantitation by high-performance liquid chromatography.<sup>32</sup> The recovery of retinol and tocopherol by this method was around 95%.

**Measurement of Free Fatty Acids.** Serum and placental homogenate lipids were extracted by the Bligh and Dyer method<sup>33</sup> and the lower organic phase was concentrated using nitrogen, resuspended in a small volume of chloroform:methanol (2:1), and used for lipid analysis. Neutral lipids were separated on silica gel G plates using the solvent system hexane:diethyl ether:acetic acid (80:20:1, vol/vol). Spots corresponding to the standard were identified by iodine exposure and eluted. Fatty acid content was quantitated using gas chromatography as described.<sup>34</sup>

**Cell Culture and Arachidonic Acid Treatment.** The Chang hepatocyte cell line is a well-differentiated nonmalignant liver epithelial cell line of human origin.<sup>35,36</sup> Cells were maintained (37°C, 5% CO<sub>2</sub>) in a growth medium (Dulbecco's modified Eagle medium; Gibco) containing 10% fetal bovine serum (Gibco), penicillin (100 units/mL), streptomycin (100 µg/mL), and amphotericin-B (250 µg/mL; Gibco). For experiments measuring reactive oxygen species and apoptosis with arachidonic acid, confluent cells were treated with various concentrations of arachidonic acid (20-100 µM) for 3 hours.

**Caspase Assay.** For detection of caspase-3 activity, hepatocytes were lysed in buffer (0.15 M NaCl, 5 mM EDTA, 1% Triton X, 10 mM Tris HCl [pH 7.4]). Caspase-3 activity was detected in cell lysates by measuring the proteolytic cleavage of the colorimetric substrate acetyl-Asp-Glu-Val-Asp (DEVD)-pNA in assay buffer (100 mM HEPES, 10% sucrose, 0.1% CHAPS [3-cholamidopropyl]dimethylammonio]-1-propanesulfonate); pH 7.5], 1 mM phenylmethylsulfonyl fluoride and 10 mM dithiothreitol) using the absorbance of released *para*-nitroanilide at a wavelength of 405 nm.

**Measurement of Mitochondrial Reactive Oxygen Species Generation in Hepatocytes.** Mitochondrial reactive oxygen species generation in cultured cells was measured by oxidation of DHR 123 to rhodamine 123. For live-cell microscopy, cells were grown in 96-well plates

**Table 1. Clinical Details of the Patients and Controls**

Details	Control (n = 10)	AFLP (n = 7)
Age	25 ± 3	29 ± 4
Gestational age (weeks)	39 ± 1	34 ± 1
Total bilirubin (mg%)	0.5 ± 0.2	12 ± 6*
Direct bilirubin (mg%)	0.2 ± 0.07	10.5 ± 5.7*
Total protein (g%)	6.3 ± 1	5.4 ± 1
Albumin (g%)	3.2 ± 0.6	2.4 ± 0.2*
AST (U/L)	17.2 ± 4.2	166 ± 66*
ALT (U/L)	12.3 ± 3	131 ± 71*
ALP (U/L)	177 ± 67	454 ± 266*
Prothrombin time-INR	0.92 ± 0.04	2.25 ± 0.6*

\*P < 0.001, when compared to controls.

and incubated with various concentrations of arachidonic acid for 3 hours in the presence of 10 µM DHR. At the end of incubation, cells were washed with phosphate-buffered saline and imaged on an inverted fluorescent microscope (Zeiss Axiovert 200M) using a rhodamine filter. Quantitation of rhodamine fluorescence was carried out in parallel experiments, where cells were lysed with lysis buffer (0.15 M NaCl, 5 mM EDTA, 1% Triton X, 10 mM Tris HCl [pH 7.4]) after incubation with arachidonic acid. Rhodamine 123 fluorescence was then measured at an excitation of 500 nm and emission of 536 nm on a spectrophotometer. The measured fluorescence value was expressed as a fold change compared to that of untreated control.

**Measurement of Lipid Accumulation in Hepatocytes.** Lipid staining with the benzophenoxazone dye Nile red was performed as follows: A stock solution of Nile red (1 mg/mL in dimethyl sulfoxide) was prepared and 1:1000 working dilution was used for staining cells. Hepatocytes in culture were treated with 20 and 40 µM arachidonic acid for 3 days, following which cells were treated with Nile red for 90 minutes. Cell nuclei was stained with Hoechst 33258, and imaged on an inverted fluorescent microscope using the rhodamine filter.

**Statistical Analysis.** Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed with the nonparametric Mann-Whitney test. Standard calculations were performed using SPSS software (version 9.0).

## Results

Clinical details for patients enrolled in the study are shown in Table 1. No differences were noted in the mean age between the two groups. Significant increase in total and direct bilirubin as well as aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and prothrombin time, accompanied by a decrease in serum albumin were noted in patients with AFLP. Microvesicular



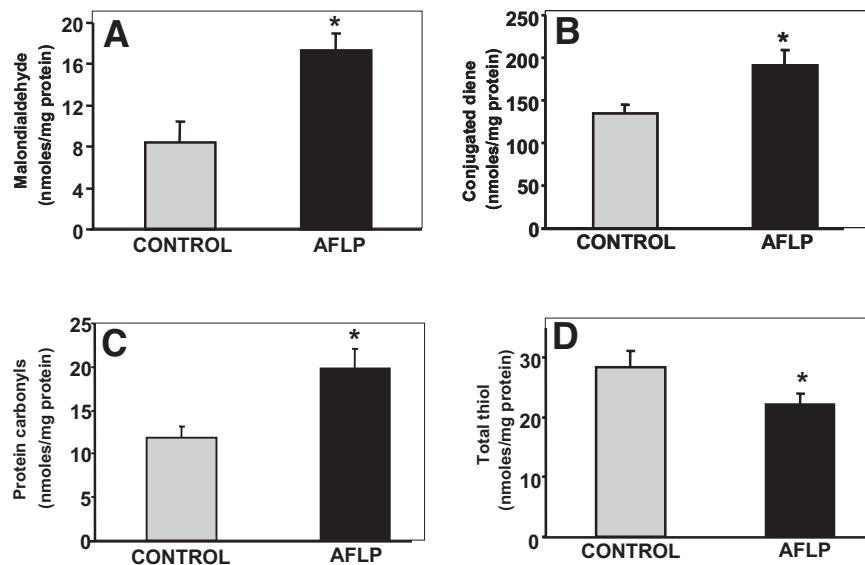


Fig. 2. Oxidative stress parameters: (A) malondialdehyde, (B) conjugated diene, (C) protein carbonyl content, and (D) total thiol in placental mitochondria isolated from patients with AFLP ( $n = 7$ ), when compared with healthy pregnant controls ( $n = 10$ ). The assays were done as described in the text. Each value represents mean  $\pm$  SD. \* $P < 0.05$ , when compared to control.

steatosis in the perivenular region was also evident on liver biopsy (Fig. 1A).

Impaired fatty acid metabolism has been suggested to play a role in the etiology of the disease and because mitochondria play a central role in fatty acid metabolism, initial experiments examined functional parameters in the placental mitochondria and the role of oxidative stress. A decrease in respiratory control ratio (Fig. 1B), along with increased mitochondrial swelling (Fig. 1C) and MTT reduction (Fig. 1D) as well as altered calcium flux (Fig. 1E,F), were seen in placental mitochondria isolated from AFLP patients as compared to controls. The calcium flux measurement shown in Fig. 1E is a representative experiment done in triplicate. Examination of oxidative stress parameters revealed a significant increase in malondialdehyde levels (Fig. 2A), conjugated diene (Fig. 2B), and

protein carbonyl concentration (Fig. 2C) along with a decrease in thiol content (Fig. 2D) in placental mitochondria isolated from patients with AFLP, suggesting oxidative stress.

Compromised mitochondrial function can lead to shunting of fatty acids to peroxisomal  $\beta$ -oxidation, and concomitant generation of reactive oxygen species.<sup>18</sup> To determine if this resulted in oxidative stress in peroxisomes, these were isolated from placenta in patients with AFLP and markers of oxidative stress were examined. An increase in malondialdehyde, conjugated diene, and protein carbonyl levels accompanied by a decrease in thiol content is evident in placental peroxisomes isolated from patients with AFLP when compared to pregnant controls (Fig. 3).

Impaired fatty acid oxidation could lead to an accumulation of fatty acids in the placenta. To determine if this

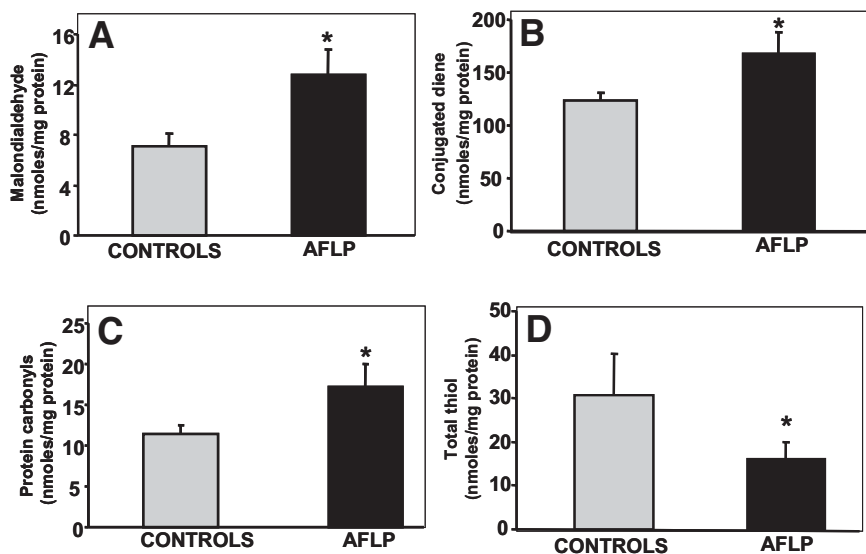


Fig. 3. Oxidative stress parameters: (A) malondialdehyde, (B) conjugated diene, (C) protein carbonyl content, (D) and total thiol in placental peroxisomes isolated from patients with AFLP ( $n = 7$ ), when compared with healthy pregnant controls ( $n = 10$ ). The assays were done as described in the text. Each value represents mean  $\pm$  SD. \* $P < 0.05$ , when compared to control.

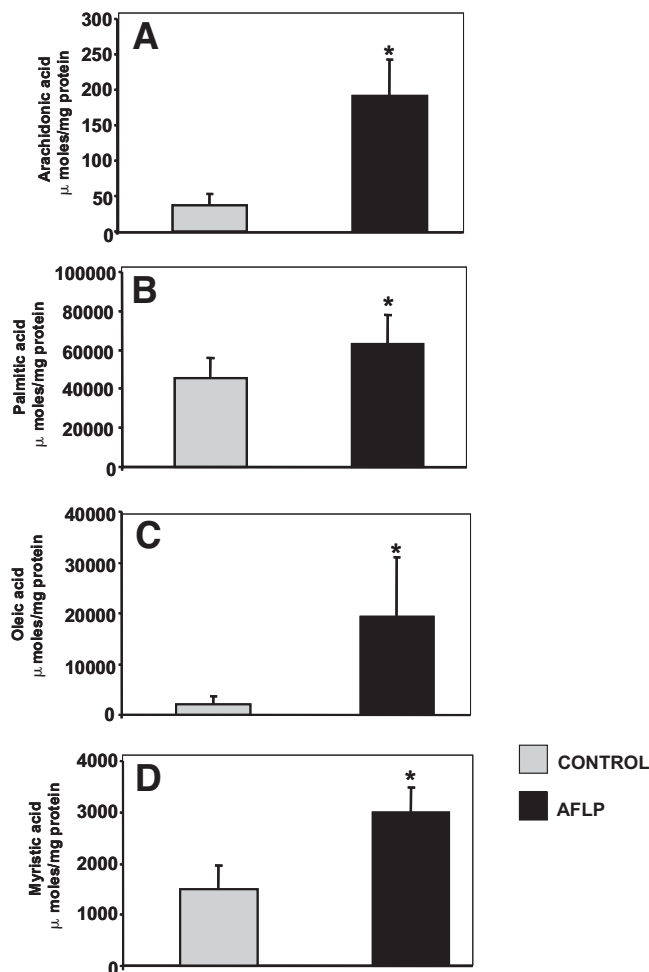


Fig. 4. Measurement of free fatty acids in placenta. (A) Arachidonic acid, (B) palmitic acid, (C) oleic acid, and (D) myristic acid from patients with AFLP ( $n = 7$ ), when compared with healthy pregnant controls ( $n = 10$ ). The assays were done as described in the text. Each value represents mean  $\pm$  SD. \* $P < 0.05$ , when compared to controls

was occurring, the levels of various fatty acids in placental tissue from patients with AFLP were analyzed. A significant increase in arachidonic, palmitic, oleic, and myristic acids was seen in placenta from patients with AFLP as compared to controls (Fig. 4).

The next series of experiments evaluated the levels of serum antioxidants and oxidative and nitrosative stress markers in maternal systemic circulation. Analysis of oxidative stress parameters in the serum from patients with AFLP demonstrated a significant increase in malondialdehyde and protein carbonyl content when compared to controls, indicating extensive lipid peroxidation and protein oxidation (Fig. 5A,B). This increase in oxidative stress markers was associated with a concomitant decrease in protein thiols (Fig. 5C). A significant increase in the levels of serum nitrate (Fig. 5D), the stable end product of nitric oxide, was also evident in serum from patients with AFLP, indicating nitrosative stress as well. These changes

were accompanied by a decrease in antioxidants such as retinol and tocopherol (Fig. 5E,F). Fatty acid analysis in serum demonstrated a significant increase in arachidonic acid and palmitic acid in patients with AFLP (Fig. 6A,B).

It has been demonstrated earlier that exposure to arachidonic acid can induce apoptosis in Hep G2 hepatocytes<sup>37</sup> by a mechanism dependent on oxidative stress.<sup>38</sup> The next series of experiments were carried out to evaluate if the arachidonic acid concentrations detected in serum of patients with AFLP could influence hepatocyte function. For these, Chang liver cells, which had been used earlier to study oxidative stress<sup>39</sup> were used as a model. Treatment of hepatocytes with various concentrations of arachidonic acid from 20-100  $\mu$ M did not result in lactate dehydrogenase release (data not shown), suggesting the absence of necrotic cell death. However, arachidonic acid induced an increase in caspase-3 activity in a dose-dependent manner (Fig. 6C) in hepatocytes, indicative of induction of apoptosis. Caspase-3 activity in the presence of 80  $\mu$ M arachidonic acid (the concentration seen in AFLP patient serum) was significantly increased from that at 20  $\mu$ M (the concentration seen in pregnant controls), suggesting that the concentration of arachidonic acid seen in serum from patients with AFLP is capable of inducing apoptosis in hepatocytes.

Mitochondrial damage has been suggested to play an important role in arachidonic acid induced toxicity,<sup>40</sup> and mitochondrial free radical generation has been implicated in the apoptotic cascade. To determine if mitochondria played a role in apoptosis in hepatocytes induced by arachidonic acid, experiments were conducted with dihydorhodamine, which has been used to detect mitochondrial free radical generation.<sup>41</sup> Treatment of Chang liver cells with arachidonic acid resulted in a dose-dependent increase in DHR fluorescence which was punctate, indicating a mitochondrial origin (Fig. 7A). Again, spectrofluorimetric quantitation indicated that exposure to 80  $\mu$ M arachidonic acid resulted in a significant increase in DHR fluorescence when compared to exposure to 20  $\mu$ M (Fig. 7B).

Acute fatty liver of pregnancy is characterized by microvesicular steatosis in the mother's liver, and we next examined if the increased arachidonic acid in serum could result in steatosis. Nile red staining of lipids has been used as an indicator of steatosis in cell culture,<sup>42</sup> and exposure of hepatocytes to 40  $\mu$ M arachidonic acid for 72 hours resulted in a significant increase in Nile red staining when compared to controls (Fig. 7C). The lipid accumulation was also punctate, with cell nuclei in the center, similar to accumulation seen in microvesicular steatosis.

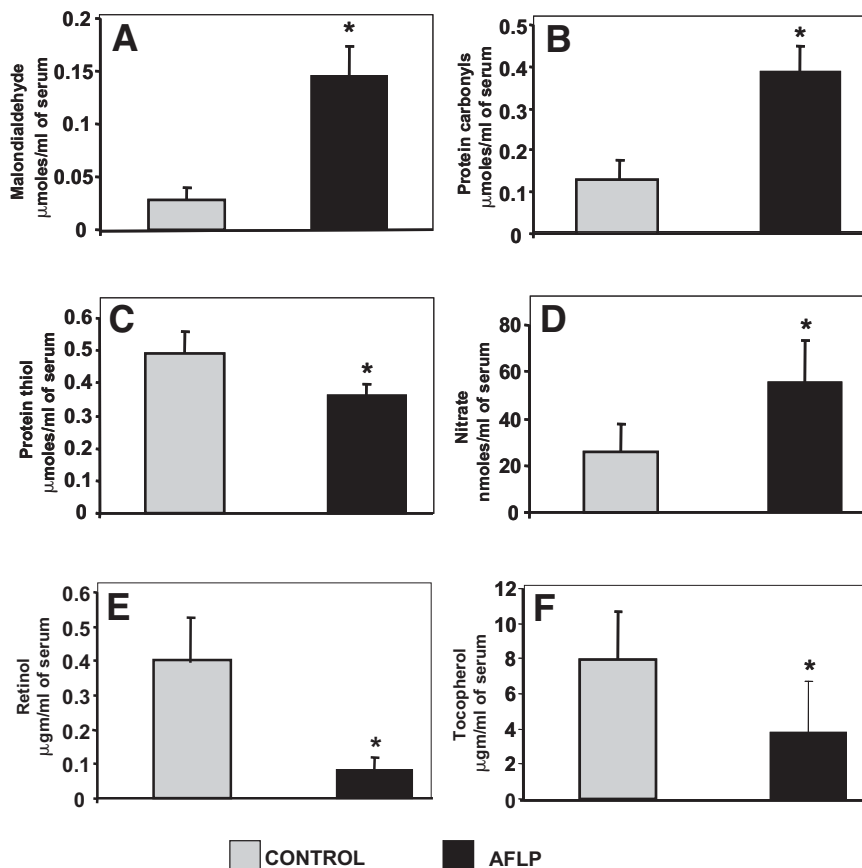


Fig. 5. Oxidative and nitrosative stress parameters and antioxidant status in serum. (A) Malondialdehyde, (B) protein carbonyl content, (C) protein thiol, (D) nitrate, (E) retinol, and (F) tocopherol from patients with AFLP ( $n = 7$ ) as compared with healthy pregnant controls ( $n = 10$ ). The assays were done as described in the text. Each value represents mean  $\pm$  SD. \* $P < 0.05$ , when compared to control.

## Discussion

Acute fatty liver of pregnancy is a sudden catastrophic illness occurring almost exclusively in the third trimester; the disorder carries significant perinatal and maternal mortality and requires early diagnosis and intervention to prevent maternal and fetal death.<sup>43</sup> Early recognition of AFLP and immediate termination of pregnancy is the key to improving maternal survival in this devastating disease.<sup>44</sup> This then led to us focusing on the role of the placenta in development of maternal liver disease. Placental mitochondria have been shown to be a source of oxygen free radicals in patients with preeclampsia,<sup>17</sup> a condition which has been suggested to be a component of the spectrum of AFLP.<sup>16</sup> Our data demonstrates that placental mitochondrial function is compromised in patients with AFLP, accompanied by oxidative stress in the organelle. In support of our findings, studies have shown that placental mitochondrial generation of superoxide could be an important source of oxidative stress in preeclampsia, leading to increased lipid peroxidation in mitochondria.<sup>17,45</sup>

In the event of compromised mitochondrial function, oxidation of fatty acids is channeled to peroxisomal  $\beta$ -oxidation, which, unlike mitochondrial fatty acid oxidation, generates hydrogen peroxide.<sup>18</sup> In addition, omega oxida-

tion of fatty acids in microsomes form long-chain dicarboxylic acids which are also a substrate for peroxisomal fatty acyl coenzyme A oxidase.<sup>46</sup> The dicarboxylic acids formed can result in increased production of hydrogen peroxide,<sup>47</sup> which can undergo the Fenton's reaction in the presence of heavy metals to form the highly reactive hydroxyl radicals and result in oxidative tissue damage. This seems to be occurring in placenta from patients with AFLP, because oxidative stress was evident in peroxisomes in addition to mitochondria. A reduced thiol content and decrease in antioxidant enzymes accompanied by increased lipid peroxidation has been shown in placentas from patients with preeclampsia.<sup>48</sup> The increased levels of arachidonic acid and other fatty acids seen in placenta from patients with AFLP raise the possibility that these changes could be due to compromised fatty acid oxidation, but further experiments will be needed to confirm this.

So how do these changes in the placenta affect the maternal liver? The data indicates significant increase in oxidative and nitrosative stress parameters in the serum and decrease in antioxidant levels. Nitric oxide is now recognized as an important molecule with wide-ranging physiological functions. The simultaneous presence of oxygen free radicals and nitric oxide can result in formation

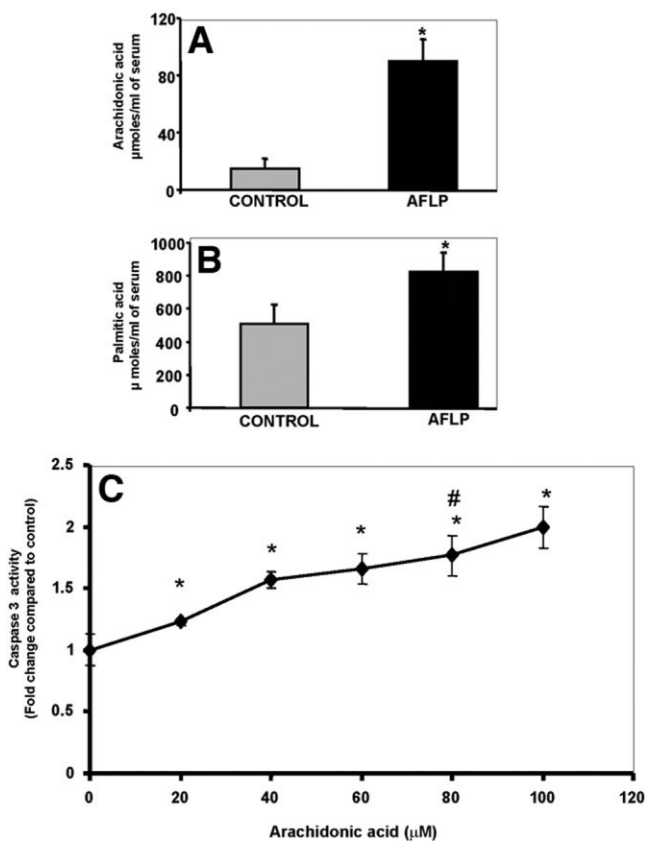


Fig. 6. Measurement of free fatty acids in serum. (A) Arachidonic acid and (B) palmitic acid from patients with AFLP ( $n = 7$ ) as compared with healthy pregnant controls ( $n = 10$ ). The assays were done as described in the text. Each value represents mean  $\pm$  SD. \* $P < 0.05$ , when compared to control. (C) Caspase-3 activity in hepatocytes treated with arachidonic acid. Confluent cells were treated with varying concentrations of arachidonic acid for 3 hours, after which cells were lysed and caspase-3 activity was determined ( $n = 3$ ). Data is expressed as mean  $\pm$  SD. \* $P < 0.05$ , when compared to control; # $P < 0.05$ , when compared to 20  $\mu$ M.

of reactive nitrogen species such as peroxynitrite,<sup>49</sup> which are highly damaging. Supplementation with antioxidants such as vitamin C and E has been shown to be beneficial in the prevention of preeclampsia,<sup>50</sup> suggesting that this is probably a global phenomenon.

In parallel to increased oxidative stress in serum, levels of fatty acids such as arachidonic acid were also found to be increased. Although none of the patients with AFLP were obese and all were from the same socioeconomic status, we cannot rule out the role of nutrition in modulating fatty acid levels. However, the increased levels of free fatty acids in the liver of AFLP patients has been suggested to be a mechanism of toxicity,<sup>51</sup> and it has also been demonstrated that exposure of hepatocytes to arachidonic acid or other polyunsaturated fatty acids results in lipid peroxidation and cellular toxicity.<sup>37,52</sup> In that case, could the elevated levels of arachidonic acid in maternal serum play a role in compromising hepatocyte

function? Arachidonic acid can induce production of reactive oxygen species from mitochondria,<sup>53</sup> and has been shown to decrease mitochondrial membrane potential, increase lipid peroxidation, and decrease cell viability in HepG2 cells.<sup>54</sup> This toxicity appears to be apoptotic in nature and was prevented by overexpression of bcl-2.<sup>37</sup> Our data with hepatocytes in culture clearly indicate that levels of arachidonic acid in patient serum can induce mitochondrial reactive oxygen species production and apoptotic cell death in hepatocytes, resulting in apoptosis. This then suggests that increased arachidonic acid and oxidative stress in serum due to placental mitochondrial dysfunction may perhaps cause hepatocyte damage in the maternal liver in patients with AFLP.

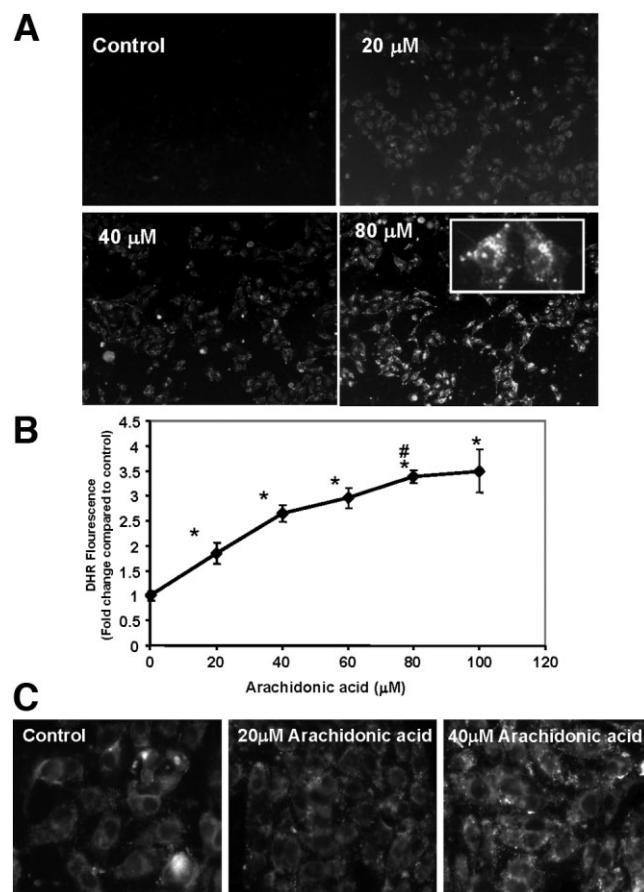


Fig. 7. (A,B) Mitochondrial reactive oxygen species generation in hepatocytes in response to arachidonic acid. Cells were treated with varying concentrations of arachidonic acid for 3 hours, following which cells were (A) subjected to live-cell imaging or (B) lysed and fluorescence measured on a spectrofluorimeter. The inset in (A) depicts the field at a higher magnification to show punctate nature of staining. In (B), data is expressed as mean  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$ , when compared to control; # $P < 0.05$ , when compared to 20  $\mu$ M. (C) Lipid accumulation in hepatocytes after exposure to arachidonic acid: Hepatocytes in culture were treated with 20 and 40  $\mu$ M arachidonic acid for 72 hours, following which cells were stained with Nile red and subjected to live-cell imaging as mentioned in the Patients and Methods section ( $n = 3$ ).



A distinctive histological feature in the liver of patients with AFLP is microvesicular steatosis, where accumulation of triglyceride occurs as small, uniform fat droplets dispersed throughout the hepatocyte.<sup>55</sup> The data so far indicate that concentrations of arachidonic acid seen in serum from AFLP patients could induce hepatocyte damage; and the data from Nile red staining, which detects triglycerides,<sup>56</sup> suggest that exposure to arachidonic acid concentrations lower than those seen in patients with AFLP over 3 days can result in an increased lipid accumulation in hepatocytes in culture similar to microvesicular steatosis in the liver.

In conclusion, this study has shown that in placenta of patients with AFLP, compromised mitochondrial function probably results in shunting of fatty acid oxidation to peroxisomes and generation of oxidative stress in these organelles. Free radical production and accumulation of fatty acids in the placenta is accompanied by oxidative stress in serum along with elevated levels of free fatty acids such as arachidonic acid. Exposure of maternal hepatocytes to this milieu probably plays an important role in mitochondrial dysfunction leading to acute liver failure. Experiments to confirm compromised fatty acid oxidation in placental mitochondria of AFLP patients and to determine levels of fatty acids in patient serum after delivery are currently underway.

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