The Antioxidant effect of Fermented Papaya Preparation Involves Iron Chelation

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Abstract

Iron-overload is a major clinical problem in various diseases. Under this condition, serum iron which surpasses the binding capacity of transferrin is present as non-transferrin bound iron and cellular unbound Labile Iron Pool (LIP) is increased. LIP participates in generation of free radicals, including reactive oxygen species (ROS). Increased ROS, with concomitant decrease in anti-oxidants, results in oxidative stress and toxicity to the liver, heart and other tissues, causing serious morbidity and eventually mortality. Therapeutic iron chelation reduces the LIP and thereby ameliorates oxidative stress-mediated toxicity. Many food-derived antioxidants have the capacities to scavenge ROS and chelate iron. We have reported that fermented papaya preparation (FPP) has ROS scavenging effect on blood cells in vitro or in vivo (in thalassemic patients and experimental animals). We now investigated FPP's iron chelating effect - its ability to prevent (and revert) LIP accumulation. Liver- and heart-derived cells, and RBCs were exposed to non-transferrin bound iron in the form of ferrous ammonium sulfate and the effect of FPP on their LIP content and ROS generation was measured by flow-cytometry. The results indicated that FPP reduces LIP and ROS, and suggest that its antioxidant mechanism is related, at least in part, to iron chelation.

INTRODUCTION

Iron overload is a major clinical problem associated with various diseases (1). In hereditary hemolytic anemias, such as thalassemia, it is caused by repeated blood transfusions and increased iron uptake in the gastrointestinal track (2). The latter is believed to be due to decreased hepcidin production associated with elevated erythropoiesis (3). In anemia of chronic disease, iron overload is due to increased production of hepcidin due to high levels of cytokines such as Il-6 (4). In other diseases such as in hereditary hemochromatosis it is caused by abnormalities in other iron-regulating proteins (5).

Normally, iron is transported in the circulation and transferred into cells through binding to transferrin (6). In iron overload, serum iron exceeds the binding capacity of transferrin, and it is present in the form of non-transferrin bound iron (NTBI) (7). In cells, iron is bound to various components such as hemoglobin, heme and cytochrome C; excess is stored in ferritin (8). In addition, all cells contain some unbound, chelatable iron, termed labile iron pool (LIP) or labile cellular iron (LCI) (9, 10). It was first suggested by Jacobs as a low molecular weight intermediate or transitory pool between extracellular iron and cellular firmly bound iron (9). This iron is redox active and it participates in generation of free radicals, reactive oxygen species (ROS) (11).

ROS have important physiological functions, but in excess they are cytotoxic due to their interaction with DNA, proteins and mainly with the membrane lipids (12). Normally, their level is tightly balanced by anti-oxidant systems that regulate the rate of ROS generation and scavenge excess ROS (13). In iron overload, increased ROS, with concomitant decrease in anti-oxidants, results in oxidative stress which affects vital organs such as the liver, heart and the endocrine system, causing serious morbidity and eventually mortality (14). In thalassemia it causes chronic anemia due to ineffective erythropoiesis (increased apoptosis of erythroid precursors in the bone marrow) and shortened survival of mature RBC in the circulation (13).

Many dietary antioxidants have the capacities to scavenge ROS (15); in addition, some of them, e.g., polyphenols, have been shown to possess iron chelating capacity (16). Fermented papaya preparation (FPP) is a product of yeast fermentation of *Carica papaya Linn*. Studies in chronic

and degenerative disease conditions, such as thalassemia(11), cirrhosis(17), diabetes (18) and aging(19), and performance sports (20) have shown that FPP favorably modulates immunological, hematological, inflammatory, vascular and oxidative stress damage parameters [for review see (20)]. We have studied its anti-oxidant properties on blood cells in various hematological diseases, such as thalassemia, paroxysmal nocturnal hemoglobinuria and the myelodysplastic syndrome (21, 22). In vitro and in vivo (in patients and experimental animals) studies showed that FPP ameliorates the oxidative stress of RBC, granulocytes and platelets. Some of these diseases are characterized by iron overload that requires chelation therapy (23). It has been shown that FPP protected against damage to DNA (induction of single and double strand breaks) and proteins (albumin) caused by combined treatment with ferric nitrilotriacetate and hydrogen peroxide, suggesting that the antioxidant properties of FPP are related to both hydroxyl scavenging as well as iron chelating properties (24).

Since LIP is the major culprit in iron-mediated cytotoxicity, in the present study we investigated the ability of FPP to prevent (and revert) cellular accumulation of LIP and thereby reduce ROS generation. Liver- and heart-derived cells, as well as RBCs were exposed to NTBI in the form of ferrous ammonium sulfate (FAS) and the effects of FPP on their LIP and ROS were measured by flow cytometry. The results indicated that FPP reduced LIP, and suggest that the antioxidant mechanism of FPP is related, at least in part, to iron chelation.

MATERIALS AND METHODS

Blood cells and cell lines. Normal human peripheral RBCs were obtained in heparin-containing tubes from normal blood donors who gave written informed consent. The research was approved by the Hadassah – Hebrew University Medical Center Human Experimentation Review Board. HEPG2 cells are human hepatoma-derived cell line (25); H9C2 cells are embryonic rat heart-derived cell line (26) (both from ATCC, Manassas, VA). The cells grew as monolayers in alpha medium supplemented with 10% fetal bovine serum, and were subcultured once a week with 0.25% (w/v) trypsin- 0.53 mM EDTA solution. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

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FPP and Iron. FPP, a product of yeast fermentation of *Carica papaya Linn,* was supplied as sachets containing 3g powder, by Osato Research Institute, Gifu, Japan. The composition of its principal components was previously reported (27). FPP was dissolved in water and used at final concentration of 50 mg/ml, unless otherwise indicated. Ferrous ammonium sulfate (FAS) (Sigma, St. Louis, MO) was freshly dissolved for each experiment in water to 1 mM and used at final concentration of 20 μM, unless otherwise indicated.

LIP and ROS. Cytosolic LIP was determined by staining the cells with 0.25 μM calcein acetoxymethyl ester (CA-AM) (Sigma-Aldrich, St. Louis, MO). CA-AM enters viable cells and becomes fluorescent upon hydrolysis by esterases; its fluorescence is quenched by binding of LIP (28). Mitochondrial LIP was measured in HEPG2 and H9C2 cells by staining with 1 μM rhodamine B-[(1,10-phenanthrolin-5-yl)-aminocarbonyl] benzyl ester (RPA, Squarix biotechnology, Marl, Germany). It enters viable cells and specifically localized in the mitochondria; its fluorescence is quenched by binding of the mitochondrial LIP (29). The CA- and RPA-fluorescence is reversely proportional to the amount of cytosolic and mitochondrial LIP, respectively. ROS were measured by staining with 0.2 mM 2'-7'-dichlorofluorescin diacetate (DCF, Sigma). Upon crossing the membrane, this compound undergoes deacetylation by cellular esterases, producing a non-fluorescent compound that is trapped inside the cells. Its oxidation by ROS produces a fluorescent compound - 2'-7'-dichlorofluorescine (30). The DCF-fluorescence is proportional to the amount ROS. Staining was done by incubating the cells with the reagents for 15-min at 37ºC.

Flow cytometry. Cell fluorescence was analyzed by a flow cytometer (FACS-calibur[R], Becton-Dickinson, Immunofluorometry systems, Mountain View, CA) as previously described (31). A 488-nm argon laser was used for excitation. At least 20,000 cells were analyzed using logarithmic amplification for the fluorescence signal height and linear amplification for forward light scatter and side light scatter. The arithmetic Mean Fluorescence Intensities (MFI) were calculated by the CellQuest[R] software (Becton-Dickinson). For each experiment, unstained cells served as controls; their MFI was <10. The results are expressed as the average ±☐ standard deviation (SD) MFI and compared using the two-sample Student's *t*-test for differences in means of MFI; p< 0.05 was considered significant.

RESULTS

The flow cytometry methodology for measuring the cellular and mitochondrial LIP contents and ROS generation is demonstrated in Fig. I. Monolayers of HepG2 cells were trypsinized and the cell suspension incubated for 2-hr in phosphate buffered saline (PBS) with FAS (20 μM) with and without FPP (50 mg/ml), or with none (Cont.). The cells were washed and stained for 15-min with either DCF (0.2 mM), CA-AM (0.25 μM) or RPA (1 μM). The cells were then washed, suspended in PBS and their fluorescence determined by flow cytometry. The results show that incubation with FAS increased the DCF-fluorescence indicating an 8.3-fold increase in ROS generation (Fig. IB); decreased the CA- and RPA-fluorescence - indicating a 3.3-fold and 9.4-fold increases in the cytosolic and mitochondrial LIPs, respectively (Fig. IC-D). FPP ameliorated these effects of iron (Fig. IB-D).

Fig. I

Using this methodology, we studied the effects of FPP on ROS and LIP in HepG2 cells, H9C2 cells and in RBCs treated with or without FAS. In these experiments (Fig. II), cells were first stained with DCF, CA-AM or RPA as indicated, washed and incubated for 1-hr with or without 20 μM FAS, FPP (50 mg/ml), both, or none (cont). Cells were then washed and analyzed by flow cytometry. The results show that in all cells tested FAS increased the DCF-fluorescence - indicating an increase in ROS generation, and decreased the CA-fluorescence - indicating an increase in the cytosolic LIP. In HEPG2 and H9C2 cells, FAS also decreased the RPA-fluorescence - indicating an increase in the mitochondrial LIP. FPP had a significant ameliorating effect on both ROS generation and LIP accumulation in cells treated or untreated with iron.

Fig. II

The dose-related effects of FAS and FPP on cytosolic LIP were studied by first staining HEPG2 cells with CA-AM (0.25 μM) for 15 min and then incubating the cells for 1-hr with the different concentrations of FAS (A) or FPP (Fig. III A and B, respectively). A decrease in CA-fluorescence indicates an increase in LIP and vice versa. The results show that addition of FAS

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drastically increased the LIP (by 290% at 50 μM and 477% at 100 μM, Fig. IIIA) while the addition of FPP moderately reduced the basal level of LIP (by 18% at 50 mg/ml, Fig. IIIB). In Figs. IIIC and D, the difference in CA-fluorescence at between each data point on the two lines indicate the effects of FPP; in Fig. IIIC, the effect of 50 mg/ml FPP in cells treated with the indicated concentrations of FAS, and in Fig. IIID, the effect of each concentration of FPP in cells treated with 20 μM of FAS. The results of these experiments show that FPP ameliorated the effect of FAS on LIP, but did not reduce it to its basal level of cells untreated with FAS.

Fig. III

The time-related effect of FPP action is demonstrated in Fig. IV. H9C2 cells were incubated with or without 20 μM FAS for 1-hr, then washed and stained for 15 min with CA-AM or DCF, washed again and incubated with or without 50 mg/ml FPP. The figure shows the changes in CA- and DCF-fluorescence at different time points after addition of FPP. The results also show that FPP ameliorated LIP and ROS starting 10 min after its addition to FAS-loaded and unloaded cells. The fact that these cells had been preloaded with FAS and washed prior to addition of FPP, suggests that components of FPP can enter the cells and chelate intra-cellular LIP.

Fig. III

The long-term effects of FPP were studied by culturing HepG2 and H9C2 cells for 3 days with FAS (20 μM), FPP (50 mg/ml), both, or none (control) (Fig. V). The cells were then washed, trypsinized and stained by DCF, CA-AM or RPA. The results show that continuous presence of FPP during the growth of the cells had a significant reducing effect on their FAS-induced cytosolic and mitochondrial LIP and consequently – their ROS (FAS+FPP vs. FAS).

Fig. IV

DISCUSSION

Iron-overload affects many organs including the liver and heart as well as erythroid cells (32-34). This toxicity is mediated by LIP-induced oxidative stress (11). In the present study, using flow cytometry methodology, we investigated the effect of FPP on the intracellular LIP, as well as on ROS, of normal RBCs and cell lines derived from heart (H9C2 cells) and liver (HepG2 cells) tissues. The results indicated that FPP reduced the cytosolic LIP in RBCs and both the cytosolic and mitochondrial LIPs in the heart- and liver-derived cells. The chelating effect was noticed minutes after addition of FPP and it continued for several days when the cells were cultured in the presence of FPP. LIP could be reduced by chelation of extra-cellular iron, but the experiments presented in Fig. IV, where the cells had been preloaded with FAS and washed prior to addition of FPP, suggest that components of FPP can enter cells and chelate intra-cellular LIP. These results suggest that FPP treatment may be preventive as well as therapeutic in the sense of being able to actively reverse iron overloaded. We have already shown that administering FPP per os to patients with thalassemia is safe and effective in reducing their oxidative stress (21, 27). The present results suggest that the antioxidant mechanism of FPP may be related, at least in part, to its ability to chelate iron.

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LEGENDS TO FIGURES

Figure I. Flow cytometry analyses of LIP and ROS in cells treated with iron and FPP. Monolayers of HepG2 cells were trypsinized and the cell suspension in PBS was incubated for 1-hr with FAS (20 μM) with and without FPP (50 mg/ml), or with none (Cont., grey). The cells were washed and diluted in PBS and labeled with for 15-min with either DCF (0.2 mM), CA-AM (0.25 μM) or RPA (1 μM). The cells were then washed and suspended in PBS and analyzed by flow cytometry. (A) A forward light scatter x side light scatter dot-plot showing a gate (R1) on live cells. (B-D) Distribution histograms of cells with respect to their DCF- (B), CA- (C) and RPA- (D) fluorescence. The mean fluorescence intensity (MFI) of each histogram is shown. The results show that iron caused an increase in the DCF-fluorescence (1000 vs. 121) – indicating an increase in ROS generation, and a decrease in CA- (279 vs. 919) and RPA- (96 vs. 904) fluorescence - indicating an increase in the cytosolic and mitochondrial LIP, respectively. FPP ameliorated these effects of iron (B-D).

Figure II. The effects of iron and FPP on cellular LIP and ROS. HEPG2 cells, H9C2 cells and RBCs were first stained for 15-min with DCF, CA-AM or RPA as indicated, washed and incubated for 1-hr with FAS (20 μM), FPP (50 mg/ml), both, or none (cont). Cells were then washed with PBS and analyzed by flow cytometry. The data are presented as the MFI (mean $\pm$ SD) of 4 experiments. The results show that in all cells tested FAS increased the DCF-fluorescence, indicating an increase in ROS generation, and decreased the CA-fluorescence, indicating an increase in the cytosolic LIP. In HEPG2 and H9C2 cells, FAS also decreased the RPA-fluorescence, indicating an increase in the mitochondrial LIP. FPP had a significant ameliorating effect ROS generation and LIP accumulation in cells treated or untreated with iron.

Figure III. The dose-related effect of iron and FPP on cellular LIP. (A-B) HEPG2 cells were trypsinized, wash in PBS, loaded with CA-AM (0.25 μM) for 15 min, wash again and suspended in PBS. Then, the cells were incubated for 1-hr with the indicated concentrations of FAS (A) or FPP (B). (C-D) Following trypsinization and washing, cells were incubated simultaneously with the indicated concentrations of FAS with ($\blacksquare$) or without ($\square$) 50 mg/ml of

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FPP (C), or with the indicated concentrations of FPP with (▲) or without (△) 20 μM of FAS (D). Following incubations, the cells were washed and analyzed by flow cytometry. The data are presented as the CA-MFI (mean ± SD) of 4 experiments. A decrease in CA-fluorescence indicates an increase in LIP and vice versa.

The results in (A-B) show that upon separate incubation, LIP was drastically increased by FAS (by 290% at 50 mM) while moderately decreased by FPP (18% at 50 mg/ml) (differences in the Y-axis in A and B should be noted). The results in (C-D) show that FPP ameliorated the effect of FPP on LIP, but did not reduce it to normal levels of cells untreated with FAS.

Figure IV. The short-term effects of FPP on LIP and ROS. H9C2 cells were incubated with or without 20 μM FAS for 1-hr, then washed and loaded for 15 min with CA-AM (A) or DCF (B), washed again and incubated with or without 50 mg/ml FPP. At different time points the cells were analyzed by flow cytometry. The data are presented as the MFI (mean ± SD) of 4 experiments. The results show that FPP increased the CA-fluorescence (i.e., decreased LIP) and decreased the DCF-fluorescence (i.e., decreased ROS) in cells loaded or unloaded with FAS.

Figure V. The long-term effects of iron and FPP on LIP and ROS. HepG2 (A-C) and H9C2 (D-E) cells were cultured for 3 days with FAS (20 μM), FPP (50 mg/ml), both, or none (Con.). The cells were then washed with PBS, trypsinized, washed again and resuspended in PBS and stained with DCF (A,D), CA-AM (B,E) or RPA (C,F) for 15 min, washed, and analyzed. The data are presented as the MFI (mean ± SD) of 4 experiments.
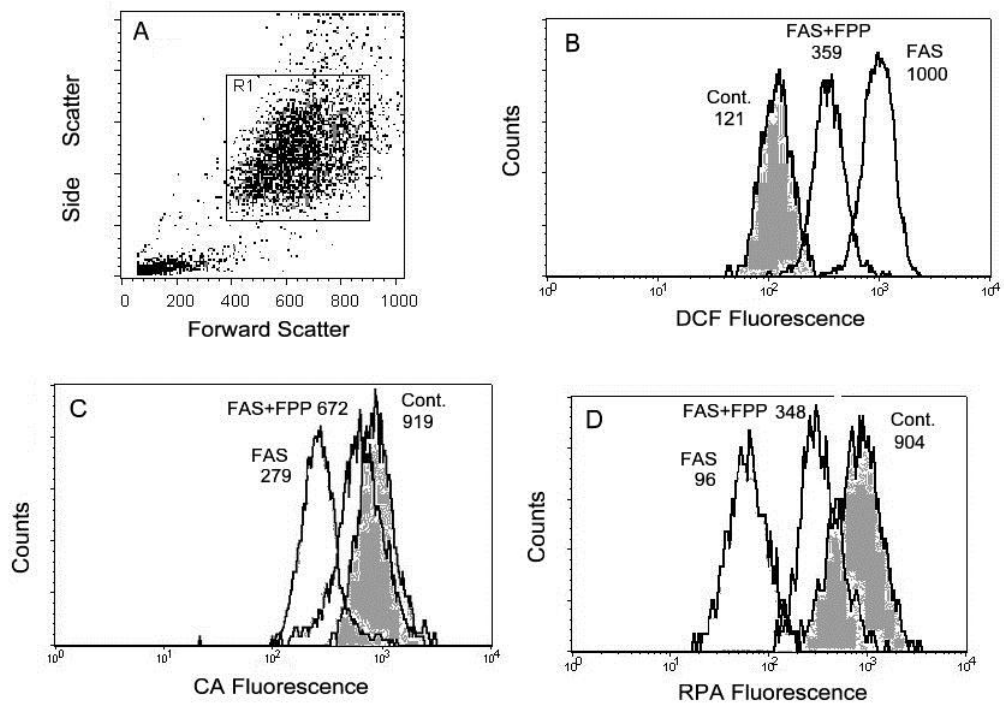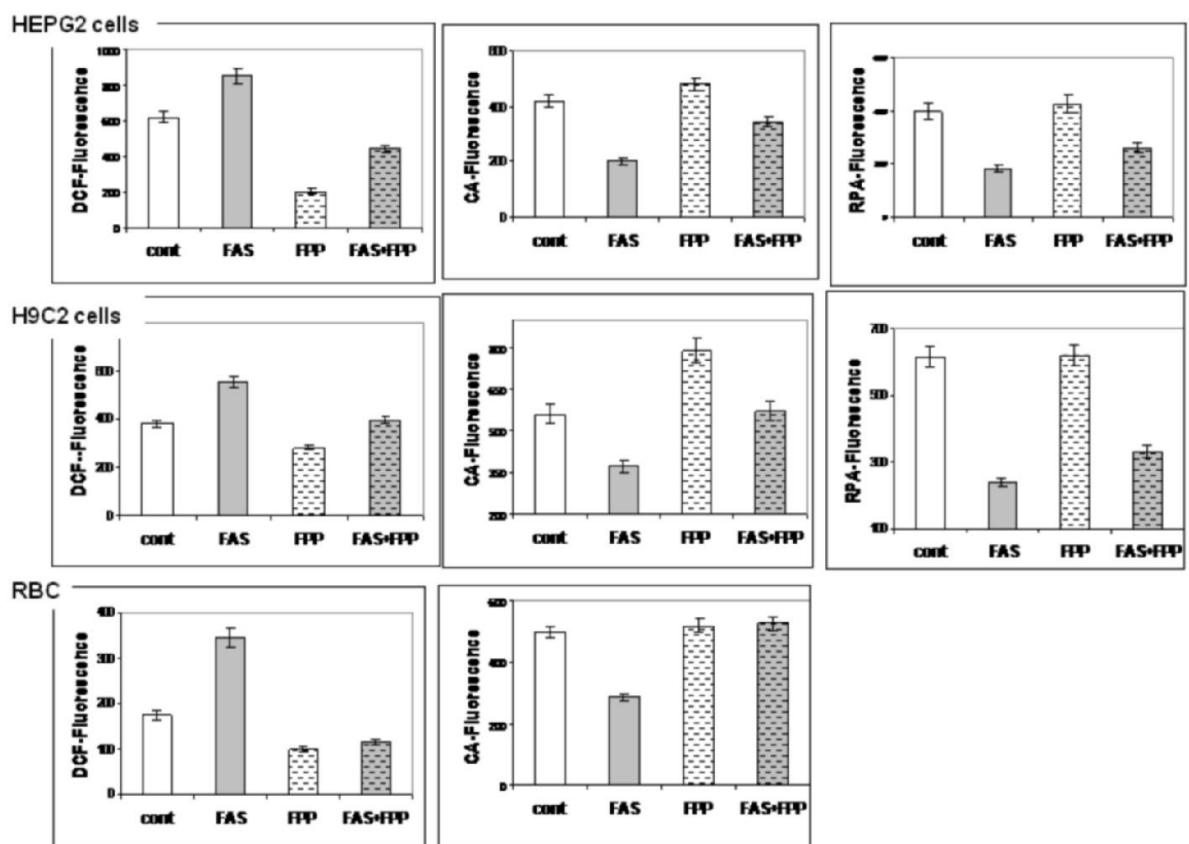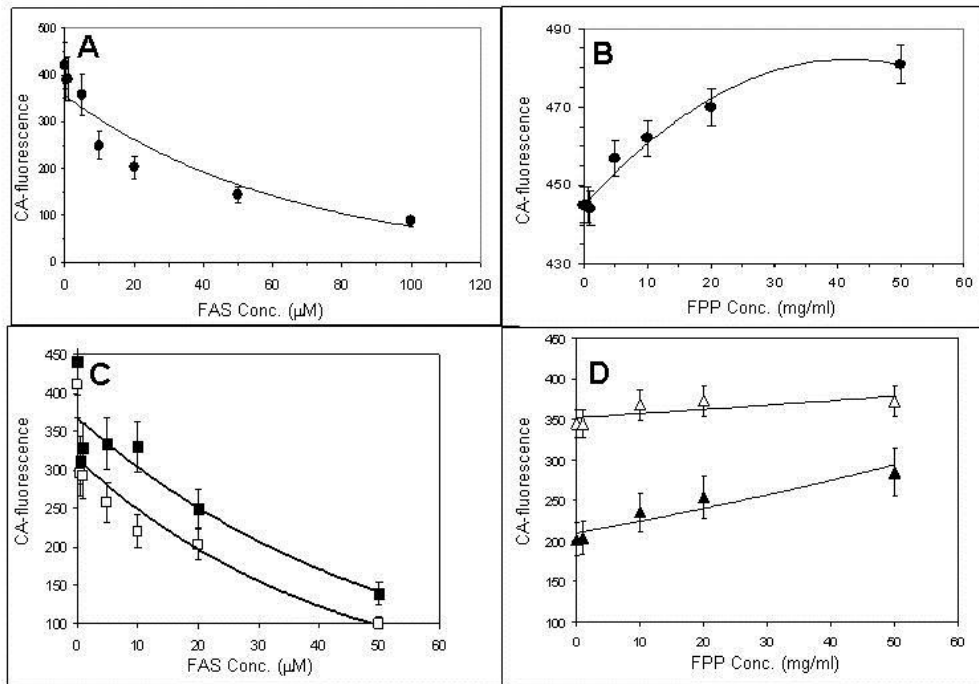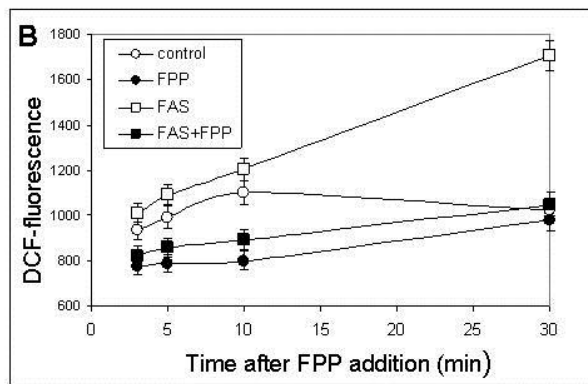
Fig. I

Fig. II

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Fig. III

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Fig. IV

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HEPG2 cells

H9C2 cells

Cont. FAS FPP FAS+FPP

Fig. V

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