Dodecanoic acid induces oxidative stress-mediated apoptotic death in liver cancer cells through mitochondrial pathway

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Research

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Abstract

Background

Dodecanoic acid (DDA), a medium chain saturated fatty acids, has been reported to have anticancer activity in reproductive system cancer and some digestive tract cancers. However, the role and the underlying mechanism of DDA in liver cancer have been rarely defined.

Methods

Mouse liver cancer Hepa 1–6 cells were administrated with DDA in this present study. Apoptosis, cell cycle analysis, mitochondrial membrane potential (MMP) and ATP content were determined by flow cytometry; GSH availability, ROS level and SOD activity was assessed by a microplate reader; Bcl-2, Bax and Caspase-3 protein levels were analyzed by western blot.

Results

0.5 mM DDA was identified as the ideal concentration for investigation and could time-dependently inhibit cell viability. DDA-treated cells had a significant, time-dependent increase in cell apoptotic rate in spite of an accumulation of the cells in S + G2/M phase of the cell cycle. The enhanced level of ROS, depletion of GSH and the reduced activity of SOD in DDA-treated cells indicated the generation of oxidative stress; mitochondrial dysfunction was evidenced by the dissipation of MMP of and the reduction in ATP content. Cell death via mitochondrial pathway was indicated by the reduced Bcl-2/Bax ratio and the increased level of caspase-3 protein.

Conclusions

Taken together, DDA effectively triggers oxidative stress-induced death in liver cancer cells by disturbing the structure and function of mitochondria. The findings provide an in-depth insight into the potential action mechanism of DDA on liver cancer.

Background

The liver is the biggest parenchymatous organ in the body, responsible for the homeostasis of many physiologic systems [1]. Liver dysfunction will cause the occurrence of a variety of liver diseases, such as fatty liver, liver fibrosis, liver cancer, and acute liver necrosis as well [2]. Liver cancer ranks the sixth highest in cancer incidence, with the second mortality in highly lethal tumors worldwide, with more than half of the total deaths happened in China, for which reason it is thus known as ‘the king of cancers’ [3]. Due to various reasons including the difficulty of early diagnosis, strong invasion and rapid development, the prognosis of liver cancer is extremely poor and its survival rate is very low [4]. Thus, how to effectively
prevent, diagnose and treat liver cancer has been becoming a critical challenge to the researchers globally now. In spite of a relatively great progress in hepatic cancer diagnosis and treatment by means of therapy including radiotherapy, chemotherapy, and surgery as well, the treatment effect is unsatisfied due to its high cost, easy reinfection, and side-effects of drug [5]. Therefore, it is urgent to find out the novel approaches, e.g., the combination of dietary and pharmacologic treatment, to deal with these problems.

The edible oil, which is an essential part in our daily diet, contains a lot of fatty acids. According to the length of the carbon chain, fatty acids are classified into three types, including short-chain (≤ 6 carbon atoms), medium-chain (7–12 carbon atoms) and long-chain fatty acid (≥ 12 carbon atoms) [6]. As one of the basic components of body, fatty acids are widely distributed in various living organisms and implicated in many physiological functions [7]. For instance, medium-chain fatty acids (MCFAs), like octanoic acid, decanoic acid and dodecanoic acid (DDA) rich in coconut oil, palm oil and milk, are the main component of cell membrane, the energy source, and even the signal molecules [8]. In recent years, many publications have reported many MCFA-caused health effects, such as improving metabolic diseases, suppressing fat accumulation, inducing cancer cell death and so on. For example, the results of St-Onge et al. suggested that medium chain triglyceride (MCT) was involved in the weight loss program without adversely affecting metabolic risk [9]. Kono and collaborators demonstrated that MCT could prevent LPS-mediated endotoxemia, a model associated to obesity and metabolic syndrome [10].

Among above MCFAs, dodecanoic acid is one of the most representative MCFA, accounting for more than 50% of total fatty acid in coconut oil with many applications [11]. For instance, Liu et al. fed 1-day-old chickens with dietary supplementary containing dodecanoic acid, and observed that growth performance, digestive ability and nutritional compounds of chicken were all improved effectively [12]. Also, there has been a body of evidence that dodecanoic acid and its derivatives show the strong antibacterial activity against gram+ bacteria through destroying their cell membranes [13]. Also, it has been reported that dodecanoic acid improves the health status of cardiovascular system because of its advantage in increasing high-density lipoproteins content and decreasing blood pressure in the rats [14]. Moreover, studies have found the anti-cancer activity of dodecanoic acid in different tumor types. For instance, using rats as an experimental animal model, Veeresh Babu and his coworkers observed that a certain dose of dodecanoic acid prevented the testosterone-induced prostatic hyperplasia [15]. Similarly, the study of Jiao et al. showed that the growth inhibitory rate of ovarian cancer cells cultured in MCFAs was significantly higher in than in the control group [16]. Furthermore, this kind of MCFA also exerted the anti-proliferative and pro-apoptotic effects on both breast and endometrial cancer cells [17]. Notably, as reported by Conceição et al., dodecanoic acid intake obviously prolonged the survival time and improved the living quality of breast cancer patients during the course of chemotherapy [18]. Besides its anti-proliferative function on reproductive cancer cells, dodecanoic acid displayed the preferential antineoplastic and oxidative stress-induced apoptotic properties in intestinal cancer cells [6]. Meanwhile, as the energy source, MCFAs could more significantly alleviate the tiredness of terminal cancer patients compared with long-chain fatty acid [19, 20].
Based on above review, the present researches on the role of DDA in cancers primarily concentrate on the reproductive cancer (i.e., ovarian cancer, prostate cancer, endometrial cancer) and colon cancer, whereas few studies concern on the effect of DDA on liver cancer. Therefore, in this study we used DDA to treat liver cancer Hepa 1–6 cells and investigated its impact on this liver cancer cells by evaluating cell viability, the cell cycle progression and apoptosis, the intercellular redox status, and the mitochondrial structure and function. Consequently, this study sheds light on the undergoing action mechanism of DDA in apoptosis induction in hepatic cancer cells to a certain extent.

**Methods**

**Cell line, chemicals and reagents**

Hepa 1-6 cell lines and Dulbecco’s modified Eagle’s medium (DMEM) were both purchased from Procell Life Science&Technology Co.,Ltd. (Wuhan, China). Dodecanoic acid (DDA) was provided by Macklin Biochemical Co.,Ltd (Shanghai, China). Fetal bovine serum (FBS) and fatty acid free-bovine serum albumin (FAF-BSA) were obtained by CellMax Co.,Ltd (Beijing, China). CCK8 was purchased from Biosharp (Hefei, China). SOD assay kit, GSH assay kit, ROS assay kit, ATP detection assay kit and mitochondrial membrane potential assay kit with JC-1 were obtained from Nanjing Jiancheng Bio-Engineering Institute (Nanjing, Jiancheng, China). BCA detection assay kit, Annexin V-FITC/PI apoptosis detection kit and DNA content assay kit were obtained from Solarbio Co. Ltd. (Beijing, China). The primary antibodies for Caspase 3, Bcl-2 and Bax were obtained from Bioworld (Wuhan, China). The secondary antibodies (goat anti-rabbit, goat anti-mouse) were obtained from Bioworld and EpiZyme, respectively.

**Cell culturing**

The Hepa 1-6 cell lines were firstly cultivated in DMEM medium containing 10% FBS after resuscitation. When reaching 70-80% confluence, the cells were digested with 0.25% trypsin and passaged routinely. The passaged cells were then seeded in the cell culture plate for DDA treatment or in the cell flask for passage under a humidified atmosphere at 37°C in 5% CO₂.

**Grouping and dodecanoic acid treatment**

Hepa 1-6 cells in logarithmic growth phase were trypsinized, harvested and seeded into 96-well plates at a concentration of 2×10⁵ cells/mL with 100 μL (each well) DMEM supplemented with 10% FBS. Following incubation of 24h, the culturing medium was replaced with 100 μL DMEM containing freshly prepared dodecanoic acid (4 mol/L), and then conjugated 0.4% FAF-BSA to final concentrations of 0.1, 0.3, 0.5, 1, 2 and 4 mM, followed by incubation for 24, 48 and 72 h to find out the optimal concentration of dodecanoic acid by CCK-8 assay. The cells treated with 0 mM dodecanoic acid were used as the control. All experimental studies were undertaken in triplicate.

**CCK-8 assay**
CCK-8 (Cell-Counting Kit-8) is now widely used in detecting cell viability on the base of WST-8, a MTT-like compound being reduced into yellow formazan dye by the dehydrogenases located in mitochondria [21]. The amount of produced formazan dye is directly proportional to the number of living cells. Briefly, after 24 h, 48 h and 72 h of administration of various doses of DDA, the cells were harvested as described above, and washed with D-PBS twice, then followed by adding 100 μL of 0.4% FAF-BSA-supplemented DMEM and 10 μL of CCK-8 per well. After 2h of incubation, a microplate reader was used to measure the absorbance value at 450 nm.

**Microscopic observation of cell morphology**

The cells were treated with the indicated concentration of DDA for 24 h, 48 h and 72 h, respectively. The morphological changes of the cells were observed under a convert microscope (SOPTOP, Japan).

**Cell cycle analysis**

The cells were seeded in a 6-well plate at a concentration of $2.5 \times 10^5$ cells/mL and cultured routinely overnight. After washing in DMEM containing 0.4% FAF-BSA twice, the cells was incubated in DMEM medium containing an indicated concentration of DDA for 24 h, 48 h and 72 h. The cells were digested, harvested and fixed in pre-cooled 70% ethanol overnight, then treated with RNase A solution at 37°C for 30 min, followed by PI staining in dark for 30 min at 4°C using a DNA content assay Kit (Solarbio, Beijing). Finally, the distribution of cell cycle was analyzed using a flow cytometry (Beckman, USA).

**Apoptosis detection**

Culturing, grouping and treatment of the cells were done as described above. Cell apoptosis was assessed by Annexin V-FITC/PI staining based on the instruction of Annexin V-FITC apoptosis detection kit (Solarbio, Beijing). In brief, the harvested cells were washed with pre-cooled PBS twice and re-suspended with 1× Binding buffer. After adjusting the cell density to $1 \times 10^6$ cells/mL, $1 \times 10^5$ cells in a volume of 100 μL were collected and mixed with 5 μL Annexin V-FITC and 10 μL PI. The cells were cultivated with the dyes for 15 min in darkness at room temperature. Thereafter, 500 μL PBS buffer was added for re-suspending the cells. After mixing, the samples were analyzed using flow cytometry for cell apoptosis rate.

**Western blot analysis**

Immunoblot analysis was carried out for assessing cell apoptosis-related proteins Bcl-2, Bax and Caspase-3. Hepa 1-6 cells were administrated with the indicated concentration of DDA for 24 h, 48 h and 72 h, respectively. After incubation, the cells were collected and lysed in RIPA solution on ice for 30 min, then centrifuged at 12000 rpm for 5 min. The supernatant containing total protein of cell extracts was collected and quantified by the BCA method at 595 nm using a microtiter plate reader. Meanwhile, the equal amounts of protein from the control or treated cells was loaded onto a 12.5% SDS-PAGE gel and
transferred to PVDF membrane. Protein blocking was performed by 5% TBST-nonfat dry milk for 2 h. Subsequently, the membranes were probed with primary antibodies against Bcl-2 rabbit antibody, Bax rabbit Ab, caspase-3 rabbit antibody (diluted 1:1000) and incubated overnight at 4°C. β-actin (diluted 1:8000) was used as an internal control. After incubated with the secondary antibody, the protein signals were detected using ECL substrate. The experiment was repeated in triplicates.

**Determination of biomarkers of oxidative stress**

**After treatment with the indicated concentration of DDA for 24 h, 48 h and 72 h at 37°C, the cells were collected to detect the related biomarkers of oxidative stress as follows:**

- **SOD activity detection**

  Briefly, the harvested cells were centrifuged at 1000 rpm for 20 min. The supernatant was discarded, and the pellet was re-suspended in PBS buffer. After incubation at 37°C for 20 min, SOD activity was determined using a microplate reader (Tecan Spark, Tecan Trading AG, Switzerland) at 450 nm, and calculated according to the instruction of SOD assay kits (Nanjing, Jiancheng, China).

- **Intracellular ROS analysis**

  The cells were trypsinized and centrifuged at 1000 rpm for 10 min, then re-suspended by D-PBS buffer. The cellular production of ROS was measured in accordance with the instructions of ROS assay kit as follows: the cells were incubated with a final concentration of 10 μM DCFH-DA reagent (Nanjing Jiancheng, China) at 37°C for 1 h, and centrifuged at 1000 rpm for 5 min. The obtained pellets were re-suspended in D-PBS, and 200 μL of the sample was added into a white microplate (Jing’an Hi-Tech, Jiangxi, China) and the fluorescent intensity of DCF was measured (Ex at 500 nm; Em at 525 nm) using multimode microplate reader. The intensity of fluorescence was proportional to ROS content, so fluorescent intensity can be used to indicate the level of cellular ROS.

- **GSH content measurement**

  Cell harvesting and rupture were undertaken as above described. The GSH level in cell extracts was determined by a GSH assay kit (Jiancheng Bioengineering Co. Ltd., Nanjing, China) following the manufacturer’s guideline. In brief, 100 μL buffer solution and 25 μL chromogenic reagent were added into 100 μL cell supernatant and then sufficiently mixed, finally the reaction was monitored at 405nm using a microplate reader. The GSH content was expressed in terms of μmol/gprot.

**Mitochondrial membrane potential (MMP) assay**
MMP was determined using a MMP assay kit (Nanjing, Jiancheng, China). In brief, Hepa 1-6 cells were administrated with the indicated concentration of DDA for 24h, 48 h and 72h, washed with pre-cooled PBS buffer, and stained using JC-1 working solution (a 1:500 dilution) for 20 min at 37°C, followed by centrifugation at 2000rpm for 5 min at room temperature. The supernatant was discarded, and cell pellets were washed and re-suspended using pre-cooled 1×incubation buffer for subsequent flow cytometric analysis. Normal mitochondria containing red JC-1 aggregates were detected with PI channel, and apoptotic cells containing green JC-1 monomer were detected with FITC channel.

**Cellular ATP level determination**

After treatment with the indicated concentration of DDA for different time at 37°C as mentioned above, the cells were harvested, ruptured by boiling and spinning at 4000 rpm for 5 min. The precipitate was removed, and supernatant extract was quantitatively analyzed for mitochondrial ATP content using a microplate reader at 636nm according to the protocol of ATP assay kit (Nanjing, Jiancheng, China). Total protein was determined by the BCA method. Cellular ATP level was expressed as a term of µmol/gprot.

**Statistical analysis**

Data are expressed as the mean ± standard deviation (SD) and all experiments were independently repeated three times. The data analysis was performed using SPSS 17.0 sofware. One-way ANOVA test was applied for analyzing multiple comparisons. $P$-value$<$0.05 was considered statistically significantly different.

**Results**

**DDA inhibits the viability of liver cancer cells**

To assess the influence of DDA on cell viability, Hepa 1-6 liver cancer cells were separately treated with 0.1, 0.3, 0.5, 1, 2 and 4 mM of DDA for 24 h, 48 h and 72 h using CCK-8 assay. As shown in Figure 1, at 24 h of treatment, 0.1, 0.3, and 1 mM of DDA didn't severely reduced the viability of Hepa 1-6 cell (93.1±4.9%, 80.0±5.2%, and 81.6±7.1%, respectively); however, 0.5 mM (70.4±2.7%), 2mM (42.9±3.5%) and 4mM (7.6±1.4%) of DDA significantly repressed cell viability when compared to the control ($p$<0.01). After 48 h of treatment with DDA at the concentration from 0.1 mM to 4 mM, cell viability showed a dose-dependent decrease (83.02±3.76%, 75.39±2.24%, 43.37±5.61%, 12.87±4.37%, 0.96±0.10%, 0.73±0.10%) compared to the control ($p$<0.01). DDA continued to greatly reduce cell viability ($P$<0.01) at 72 h postincubation compared to the control, the most obvious of which was at 1 mM, 2 mM and 4 mM with 2.7±0.5%, 1.9±0.6% and 0.8±0.3%, respectively (Table 1). The result showed that, in spite of the lower cell viability in each dosage group than in the control, DDA of <0.5mM had the relatively weak inhibition effect on liver cancer cell growth, whereas high concentration of more than 0.5 mM had the strong toxicity to the cells.
Therefore, a concentration of 0.5 mM DDA was considered as the optimum concentration for further study.

To further test the effect of DDA on cell morphology, Hepa 1-6 cells were treated with 0.5 mM DDA and the morphological changes were observed under an inverted microscope. As shown in Figure 2, the cells in the control group exhibited an adhesive morphology characterized by the uniform cell distribution and good growth, while DDA-treated cells showed the severe changes in morphology characteristically associated with cell death (e.g., the appearance of floating cells) and growth inhibition (e.g., the decrease in cell population). The longer the treatment time, the more severe the cell morphological alterations were.

**DDA might induce the block of cell division in Hepa 1-6 cells**

The cell cycle distribution of Hepa 1-6 cells after administrated with 0.5 mM DDA was analyzed using the flow-cytometric assay and the result was shown in Figure 3A (left panel). Compared to the control, the percentage of G1-phase cells in the treated groups were slightly increased at 24 h (50.53%) of treatment (p>0.05), whereas significantly reduced to 34.50% at 48h, and 23.77% at 72 h (p<0.01). The percentages of S-phase cells in DDA-treated groups at 48 h (24.98%) and 72 h (22.57%) were both remarkably higher than that in the control (13.99%, p<0.01) except for 24h. On the contrary, the percentage of G2/M-phase cells greatly decreased from 21.92% in the control to 9.89% in 48h-treated group, and to 7.56% in 72h-treated group in a time-dependent manner (p<0.01).

However, sub-G1 populations were higher in the DDA-treated groups than the control group (3.14%), even reaching the highest point 34.50% at 72 h (p<0.01, Fig. 3B). Above data indicated that the block in Hepa 1-6 cells in response to DDA didn't appear at any phase of the cell cycle, implying that DDA maybe restrains the process of division of nucleus or/and cytoplasm.

**DDA induces the apoptotic death in Hepa 1-6 cells**

To further determine whether the decrease in cell viability observed in Hepa 1-6 cells treatment with DDA was the result of apoptotic induction, Annexin V-FITC/PI based on flow cytometry analysis was applied to estimate the cell apoptotic rate. The result was shown in Figure 4 that indicated a time-dependent increase (p<0.01) in total apoptotic rate of DDA-treated cells in contrast to the untreated cultures. Moreover, as displayed in Figure 4E, the percentage of DDA-untreated cells distributed in early apoptosis was very low with only 2.86%; at 24h in the presence of 0.5 mM of DDA, the apoptotic rate was just only increased to 3.53%. Statistical analysis showed no significant difference (p>0.05) in cell apoptotic rate between these two groups. Whereas, the percentage of Hepa 1-6 cells in early apoptosis rose to 17.37% at 48 h and 21.70% at 72 h, respectively, showing the significant alteration in apoptotic rate (p<0.01).

**DDA induced oxidative stress in hepa 1-6 hepatic cancer cells**

This study investigated the biomarkers of oxidative stress including cellular ROS production, GSH content, and SOD activity in Hepa 1-6 cells following treatment with 0.5mM DDA for different time. The production of ROS and GSH in Hepa 1-6 cells after DDA treatment was evaluated using the corresponding
assay kit. As shown in Figure 5A, a time-dependent increase in ROS level was detected in Hepa 1-6 cells treated with DDA. Contrarily, a decrease in GSH content was observed in the DDA-administrated cells although not gradual reduction with the time extended (Fig. 5B). According to the statistical analysis, ROS and GSH levels after treatment with DDA had a significant difference (p<0.01) compared with the control. Next, the activity of antioxidative enzyme SOD was estimated by a SOD assay kit. Compared with the control group (12.01 U/mgprot), SOD activity showed a trend toward reduction in DDA-treated cell cultures with the prolongation of treatment time (Fig. 5C) and there was a statistically significant decrease (p<0.05) in SOD activity only at 72 h (9.23 U/mgprot).

**DDA impacts the mitochondrial membrane potential (MMP) of Hepa 1-6 cells**

MMP is an important bio-marker indicating the integrity of mitochondrial structure, and its changes in the cells treated with 0.5 mM of DDA for different time periods were measured by the JC-1 assay using flow cytometry. As shown in Figure 6, the cells in the control group had the relatively high MMP levels; as compared with the untreated control, the mitochondria in DDA-treated cells had the lower MMP level and less accumulation of JC-1 for J-aggregate formation (Figure 6A). Furthermore, Figure 6B demonstrated that MMP could be effectively dissipated (p<0.01) by 0.5 mM of DDA in a time-dependent manner.

**DDA reduces the generation of mitochondrial ATP in Hepa 1-6 cells**

The effect of DDA on the production of mitochondrial ATP in Hepa 1-6 cells was evaluated using ATP determination kit and the result was demonstrated in Table 2. In contrast to the control cultures (939.31 µmol/gprot), mitochondrial ATP values in the cells were reduced after treatment of DDA which caused a significant time-dependent depletion of ATP with 877.15µmol/gprot at 24 h (p<0.05), 790.55mol/gprot at 48 h (p<0.01) and 513.81µmol/gprot at 72 h (p<0.01) when compared with the control group.

**DDA modulates the expression of apoptotic-related proteins Bcl-2, Bax and Caspase-3**

To further explore whether death-promoting effect of DDA was related to intrinsic mitochondrial apoptotic pathway, we conducted western blotting to detect the expression of proteins including Bcl-2, Bax and Caspase-3. As shown in Figure 7A, administrating DDA into Hepa 1-6 cells increased the expressions of pro-apoptotic Bax and Caspase-3, while decreased the expression of anti-apoptotic Bcl-2 in a time-dependent manner. A further quantification analysis of grey-scale intensity value indicated that levels of Bax and Caspase-3 proteins were both significantly higher (p < 0.01) in DDA-treated Hepa 1-6 cells than in the untreated cells (Fig. 7B). Whereas, the expression of Bcl-2 was significantly decreased (p< 0.01) in the cells treated with 0.5 mM of DDA (Fig. 7B).

**Discussion**

As one of 12-carbon saturated medium chain fatty acids, dodecanoic acid is one of important constituent of many vegetable oils, especially like coconut oil and palm-kernel oil [22]. Nowadays, this fatty acid has been extensively used in many industries, e.g., Food, Medicine, and Daily Chemical etc [23, 24]. With the
deepening of the related researches, DDA has been found to exert a marked inhibitory effect on the growth of some tumor cells like prostate cancer, breast cancer, stomach cancer and lung cancer [25–27]. However, the anticancer effects of DDA against liver cancer cells remain unclear.

In the present study, liver cancer Hepa 1–6 cells was used as experimental subjects and administrated with DDA at different concentrations ranging from 0.1 mM to 4.0 mM for different time periods. As a result, various concentrations of DDA exerted, to different extents, the inhibition effect on cell viability. However, the influence of DDA < 0.5 mM was relatively poor, while DDA with higher than 0.5 mM showed a cytotoxic effect against the cells. For this reason, we selected 0.5 mM of DDA as the suitable concentration for attempting to unravel the anti-liver cancer mechanism of DDA by the in vitro study. Also, it was observed under the microscope that 0.5 mM DDA could induce the morphological alterations characteristically related to the inhibition of cell growth.

In addition, the recent findings suggested that dodecanoic acid could induce oxidative stress of colon cancer cells in vitro [6]. As a consequence, it could be hypothesized that oxidative stress induced by DDA caused the cell division arrest and apoptosis. In this study, oxidative stress induced by 0.5 mM of DDA was indicated by the increase of intercellular ROS level, depletion of GSH, and the reduced activity of antioxidant enzyme SOD. In accordance with the study by Fauser et al [6], our findings showed that DDA time-dependently increased the level of cellular ROS, whereas decreased the GSH level in parallel. Functionally, GSH has been well-recognized as one of the most important non-enzymatic members regulating redox balance in the anti-oxidation system and can directly clean reactive oxygen-derived free radicals [28]. One of the reasons for the increase in ROS level might be that the excessively produced ROS in the DDA-administrated cells couldn't be scavenged timely due to the depletion of GSH. Another one could be that the enzymatic anti-oxidation system is involved in balance of intracellular oxidation/reduction status by scavenging various ROS, such as superoxide anion, hydrogen peroxide and so on, among which SOD is one of key enzymatic antioxidants for helping the removal of free radicals [29]. And the findings that DDA significantly reduced the SOD activity in the cells perhaps might be another potential cause of ROS level elevation after DDA treatment.

In general, the production of oxidative stress beyond the capacities of antioxidant mechanisms leads to cell division arrest for repairing the damaged DNA, or triggers apoptotic cell death [30]. Thus, the present study further analyzed cell cycle distribution and cell apoptosis after treated with DDA. It was found that the percentage of cells distributed in S phase was greatly increased, while that of the cells in both G1 and G2/M phases was severely reduced. The reason might be related to the differences in the experimental environment, experimental operation procedure and the experimental subject, which needs to be further verified by other experimental means. These observations were obviously different from the previous report that dodecanoic acid reduced the counts of colon cancer cells in G1 phase, while maintained them in S and G2/M phases of the cell cycle [6]. However, what's interesting was that DDA significantly induced the apoptotic death in Hepa 1–6 cells as evidenced by Annexin V-FITC/PI double staining combined with flow cytometry analysis, which was in accordance with Fauser's report on the effect of DDA on colon
cancer cell apoptosis [6]. It might be speculated that dodecanoic acid preferentially impacted the mitosis or cytokinesis rather than DNA replication in liver cancer cells.

Many anti-tumor natural substances have been proven to induce apoptotic death in cancer cells through the mitochondrial pathway [31, 32]; however, whether DDA causes cell death via this pathway is still unclear. Now, it is popularly thought that death via the mitochondrial pathway is modulated by Bcl-2 family [33]. Under normal conditions, the pro-apoptotic protein Bax is located in the cytoplasm and controlled by the anti-apoptotic Bcl-2 [34]. In detail, the presence of apoptosis-related stimulus induces the mitochondrial translocation of Bax, decreases the ratio of Bcl-2/Bax, changes the mitochondrial membrane permeability, triggers Cyto C release and Caspase activation, and ultimately leads to cell death[35]. Based on above description, it can be inferred that the adjustment within the Bcl-2 family is one of the main characteristics of apoptosis via the mitochondrial pathway. In consistent with the previously measured apoptotic parameters, the result from western blot analysis showed an increase in Bax protein level while a decrease in Bcl-2 protein level in DDA-treated cells. In addition, as an important biomarker in mitochondrial apoptotic pathway, a time-dependent increase in Caspase-3 protein level was also observed in Hepa 1–6 cells after treatment of DDA.

As reported in the previous works by other researchers, the constant production of ROS induced by oxidative stress firstly damages the mitochondrial structure, disrupts the mitochondrial membrane potential (MMP) and causes mitochondrial dysfunction, suggesting the hallmark role of MMP change in mitochondrial apoptosis [36, 37]. Also, MMP dissipation has been widely accepted as one of the hallmarks of mitochondrial apoptosis [38]. Here, we found the destructive effect of DDA on mitochondria structure integrity, which was evidenced by the observations that MMP was time-dependently depolarized in Hepa 1–6 cells after administration of DDA. In addition, our result also showed that the change in MMP was accompanied with a significant decrease in ATP content in parallel. These findings suggested that the mitochondrial structural and functional damage induced by this kind of fatty acid was correlated with the generation of oxidative stress in DDA-treated cells.

**Conclusions**

Due to the limitation of the in vitro experiment, the present study only presented the primary evidence of anti-tumor activity of DDA and the possible action mechanism in liver cancer cells. Based on the data from this study, it might be concluded that dodecanoic acid induced the oxidative damage to liver cancer cells, thus triggering mitochondrial dysfunction and the subsequent cell apoptotic death via the mitochondrial pathway. In detail, DDA perturbs the redox balance in the cells, representing the characteristics of GSH reduction, ROS over-generation, and inhibition of SOD activity. The generation of oxidative stress exceeding the range of capacity of cellular antioxidant system damages to the cells, causes the structural and functional destruction of mitochondria, thus initiating cell death via mitochondrial pathway (Fig. 8).
Despite the preliminary understanding on the pro-apoptotic role of DDA in hepatic cancer cells, there are still some questions to be answered with respect to how this fatty acid causes oxidative damage to liver cancer cells? Whether it disturbs the cell cycle progression or cytokinesis/nucleus division on earth? These issues need to be solved by means of the in-depth researches in future.

**Abbreviations**

MCFAs: Medium-chain fatty acids; DDA:Dodecanoic acid; MCT:Medium chain triglyceride; DMEM:Dulbecco's modified Eagle's medium; FBS:Fetal bovine serum; FAF-BSA:fatty acid free-bovine serum albumin; CCK-8:Cell-Counting Kit-8; MMP:Mitochondrial membrane potential; SD:Standard deviation

**Declarations**

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**Authors' contributions** Xiaoguang Chen and Liwei Guo designed the project and performed the experiments. Yumei Liu analysed the data. Xuemin Zhu designed the project. Qiongxia Lv wrote the manuscript.

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**Availability of data and material** All Materials used in this study and data generated or analyzed during this study are included in this article.

**Ethics approval** All experiments and methods were performed in accordance with the relevant approved guidelines and regulations, as well as under the approval of the Medical Ethics Committee of the First Affiliated Hospital of Henan University of Science and Technology.

**Consent to participate** Not applicable

**Consent for publication** I declare that all authors have approved the submission of this manuscript to this journal and publication on this publication.

**Competing interests** The authors declare that no competing interests exist.

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Tables

Table 1. The effect of different dosages of dodecanoic acid on the survival rate (%) at different times after treatment

<table>
<thead>
<tr>
<th>Dodecanoic acid (mmol/L)</th>
<th>Cell viability (%) at different treatment time</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>24h</td>
</tr>
<tr>
<td>0</td>
<td>100.00±8.97\textsuperscript{Aa}</td>
</tr>
<tr>
<td>0.1</td>
<td>93.11±4.86\textsuperscript{Ab}</td>
</tr>
<tr>
<td>0.3</td>
<td>79.88±5.23\textsuperscript{Bb}</td>
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<td>0.5</td>
<td>70.40±2.75\textsuperscript{C}</td>
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<tr>
<td>1</td>
<td>81.61±7.13\textsuperscript{Bb}</td>
</tr>
<tr>
<td>2</td>
<td>42.91±3.48\textsuperscript{D}</td>
</tr>
<tr>
<td>4</td>
<td>7.56±1.39\textsuperscript{E}</td>
</tr>
</tbody>
</table>

Note: Within the same column, different lowercase letters and capital letters within the same column mean significant difference ($P<0.05$) and extremely significant difference ($P<0.01$), respectively; the same
letters represent no significant difference ($P > 0.05$)

Table 2. Changes in ATP levels after cells administrated to 0.5 mM of DDA for different time periods.

<table>
<thead>
<tr>
<th>Grouping</th>
<th>ATP level (µmol/gprot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>939.31±18.25$^{Aa}$</td>
</tr>
<tr>
<td>24h treatment</td>
<td>877.15±23.99$^{Ab}$</td>
</tr>
<tr>
<td>48h treatment</td>
<td>790.55±29.85$^{Ba}$</td>
</tr>
<tr>
<td>72h treatment</td>
<td>513.81±34.15$^{Ca}$</td>
</tr>
</tbody>
</table>

**Note:** Within the same column, different superscript lowercase letters and capital letter mean significant difference ($P < 0.05$) and very significant difference ($P < 0.01$), respectively; the same superscript letters represent no significant difference ($P > 0.05$).

**Figures**

![Graph showing cell viability over time for different DDA concentrations](image)

Figure 1
CCK-8 result showing the effect of different doses of DDA on the viability of hepa1-6 cells.

Figure 2

The images of observation under an inverted microscope after different time with 0.5 mM DDA (scale bar=200 μm).
Figure 3

The cell cycle distribution of Hepa 1-6 cells treated with DDA for 24 h, 48 h and 72 h. Columns not sharing the same superscript capital letters differ significantly (p< 0.01).
Figure 4

Apoptosis analysis of Hepa 1-6 cells induced by DDA for the indicated time periods. Cells were treated with DDA at concentrations of 0.5 mM for 24 h, 48 h and 72 h, and stained with Annexin V-FITC/PI using flow cytometric analysis. Note: (A) control (untreated); (B) 24 h; (C) 48 h; (D) 72 h; (E) Schematic diagram showing the apoptotic rates of the cells. Columns not sharing the same superscript capital letters differ significantly (p< 0.01).
Figure 5

Reactive oxygen species (ROS) content, glutathione (GSH) content and superoxide dismutase (SOD) activity for each group (mean ± SD). Note: Compared with Control group; columns not sharing same superscript capital letters differ significantly (p < 0.01).
Figure 6

The changes in MMP level of Hepa 1-6 cells after treated with DDA. (A) Typical MMP patterns of DDA-treated Hepa 1-6 hepatic cancer cells. The cells were treated with 0.5 mM DDA for 24 h, 48 h and 72 h, and subjected to measurement of MMP by the JC-1 assay. (B) The ratio of red to green fluorescence for (A). Bars with different superscript capital letters represent statistically significant difference (p< 0.01).
Figure 7

Expressions of Bcl-2, Bax and caspases 3 in Hepa 1-6 cells with DDA treatment. Cells were treated with 0.5 mM DDA for 24 h, 48 h and 72 h. (A) Western blots of proteins in control and DDA-treated Hepa 1-6 cells. DDA treatment significantly enhanced Bax and Caspase-3 expression, and inhibited Bcl-2 expression in a time-dependent manner. β-actin was an internal control. (B) Grey-degree value analysis of DDA-induced apoptotic proteins in Hepa 1-6 cells. Data was expressed as the means of three independent
experiments ± SD. Columns not sharing same superscript letters within the group differ significantly (p < 0.01).

Figure 8

The schematic representation of the possible action pathway of DDA-inducing liver cancer liver. DDA perturbs the redox balance which causes oxidative stress damages to the structural and functional destruction of mitochondria, thus initiating cell death via mitochondrial pathway.