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Study of impact of *Wolbachia* surface protein on reactive oxygen species production in HepG2 cell line exposed to ethanol

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ABSTRACT

Objective: To study the impact of *Wolbachia* surface protein (WSP) on reactive oxygen species (ROS) level in ethanol (EtOH)-exposed HepG2 cells.
Materials and Methods: Increase in ROS level was induced in HepG2 cells by subjecting HepG2 cells to EtOH exposure. Impact of WSP on ROS level was examined by staining of intracellular ROS in cells using the specific ROS-detecting dye 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCFDA), followed by flow cytometric analysis.
Results and Conclusion: Flow cytometry analysis using H2DCFDA-based staining study of ROS level in HepG2 cells revealed that EtOH caused oxidative stress in HepG2 cells by inducing production of high levels of ROS. However, EtOH-induced increased ROS production in cells decreased with treatment of WSP.
From the current study, we can culminate that WSP provides cytoprotective action against EtOH-induced increased ROS production and oxidative stress in HepG2 cells by decreasing ROS production. This will be of significance for the treatment of EtOH-related liver ailments. Thus, this article emphasizes that WSP with protecting ability could be used as a powerful therapeutic drug to treat EtOH-related liver ailments.

1. INTRODUCTION

Wolbachia surface protein (WSP) is the most plentifully expressed protein of *Wolbachia,* a Gram-negative endosymbiotic bacteria. Former research demonstrated that rWSP leads to apoptosis of monocytes and not lymphocytes [1]. Another article by Bazzocchi et al. [2] has demonstrated that WSP, a surface protein of the endosymbiotic bacteria from filarial nematodes possess anti-apoptotic activity by retarding apoptosis in human polymorphonuclear cells PMNs. Inspiring results of the previous studies stimulated us to examine the impact of WSP on ROS production and oxidative stress in HepG2 cells exposed to ethanol (EtOH). Although there is information on the impact of WSP on different varieties of human cells, however, the information on the impact of WSP (got from *Wolbachia* of *Exorista sorbillans*) on EtOH produced toxicity in reference to flow cytometric study of ROS production and oxidative stress in HepG2 cells is not found out yet.

Reactive oxygen species (ROS) are very reactive, short-lived molecules formed by incomplete one-electron depletion of oxygen. ROS are oxygen consisting molecules having unpaired electrons. They are produced as a result of cellular metabolism [3,4]. ROS include superoxide anion radical (O_2•-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), singlet oxygen ($^1O_2$), peroxyl radical (ROO•), etc., [5–7]. ROS are produced as part of the body's usual metabolic process and by external stimuli like heavy metals and other toxicants [7]. Keeping the proper quantity of intracellular ROS is necessary for maintaining the redox stability and signaling cellular proliferation [8]. ROS usually occur in balance by biochemical antioxidants in all aerobic cells [9]. Quantity of ROS increases more than usual level either because of enhanced ROS production or decreased antioxidant ability [10]. Enormous production of ROS in cells causes oxidative stress [11]. Oxidative stress caused because of excessive accumulation of ROS results in damage of cellular components, including DNA, proteins, and lipids [12,13]. Excessive ROS production is an indicator of oxidative stress [14]. Flow cytometry can be utilized for detecting ROS using fluorescent probes [15–19]. So, here in this study, we have investigated and interpreted the impact of WSP

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on the ROS level in HepG2 cells exposed to EtOH by determining the ROS levels by means of flow cytometer.

As WSP will be resulting from *Wolbachia*, the impact of WSP might possibly differ relying on the nature of *Wolbachia* used that which may possibly rely on the origin of *Wolbachia* and the impact of WSP might also possibly differ relying on the type of the cells that which WSP comes into contact. Hence, here in this article, WSP, the protein of *Wolbachia* bacteria got from *E. sorbillans* was utilized. HepG2 cells were taken and utilized for examining the impact of WSP in HepG2 cells exposed to EtOH, since HepG2 cell line: i) is obtained from human liver tissue, ii) has the original phenotype of human liver tissue, iii) powerful antioxidant systems which could easily protect against ROS that are generated in its metabolic pathways, and iv) is also used to investigate whether the substance is cytotoxic or cytoprotective [20–23]. EtOH was utilized in this study to produce excessive ROS as it is known from the literature that: i) acute and chronic EtOH treatment has been exhibited to enhance the generation of ROS, decrease cellular antioxidant levels and increase oxidative stress in numerous tissues, particularly the liver. EtOH-induced oxidative stress plays an important function in the process by which EtOH causes liver injury, ii) EtOH metabolism causes the generation of ROS, and iii) it is also recognized that EtOH produces toxicity in HepG2 cells [24–27].

Considering each one of the points mentioned above, we were intrigued to examine the impact of WSP (got from *Wolbachia* of *E. sorbillans*) on ROS production and oxidative stress in HepG2 cells exposed to EtOH. Hence, here in this article, the impact of WSP on ROS level in HepG2 cells exposed to EtOH was investigated by means of flow cytometry.

In this article, we studied the impact of WSP on ROS in HepG2 cells in reaction to EtOH exposure. To investigate the impact of WSP, increase in ROS level and oxidative stress was induced in HepG2 cells by means of exposure of HepG2 cells to EtOH. ROS level in HepG2 cells was measured by 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCFDA) staining followed by flow cytometry analysis. ROS level was analyzed in cells on the basis of H2DCFDA staining. H2DCFDA staining determines ROS on the basis that H2DCFDA/DCFH-DA, a lipid-soluble, membrane permeable, primarily non-fluorescent compound get cleaved by intracellular non-specific esterases to eliminate the diacetate part and form the membrane impermeable non-fluorescent product, dichlorodihydrofluorescein (H2DCF). H2DCF is oxidized by intracellular ROS to generate a highly fluorescent compound 2′, 7′-dichlorofluorescein (DCF) which is a measure of the quantity of ROS present in the cells that emits green fluorescence [15,18,19,28–32]. The DCF fluorescence which indicates intracellular ROS level [18,19,28] was determined in H2DCFDA stained HepG2 cells by flow cytometer [FACSCalibur Becton Dickinson (BD) Biosciences, San Jose, CA]. Flow cytometric evaluation of H2DCFDA staining analysis for labeling of ROS exhibited that EtOH induced increase in ROS level and oxidative stress in HepG2 cells and the percentage of this EtOH-induced increase in ROS level and oxidative stress in the cells were reduced in the presence of WSP by reducing the ROS level. The cytoprotective impact of WSP was like that of Silymarin, which has hepatoprotective and antioxidative properties [33]. Therefore, these outcomes demonstrated that treatment with WSP defended cells against the increase in ROS level and oxidative stress by lessening the ROS. Thus, the present data demonstrate that WSP has the capability to protect against an increase in ROS level and oxidative stress in EtOH-exposed HepG2 cells. The current data suggest that due to the protecting impact of WSP, it could function as likely beneficial therapeutic drug in the treatment of EtOH-related liver ailments.

2. MATERIALS AND METHODS

2.1. Chemicals and materials

H2DCFDA, Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), L-glutamine, Penicillin, Streptomycin, Trypsin-Ethylenediaminetetraacetic acid (EDTA), Phosphate Buffered Saline (PBS), and Dulbecco's Phosphate Buffered Saline (DPBS) were bought from Sigma Chemical Company, St. Louis, MO, USA. Cell culture plasticwares were purchased from Fisher Scientific (Pittsburgh, PA).

If not specified all other chemicals, reagents and materials used in this work were purchased from Sigma Chemical Company, St. Louis, MO. All other chemicals used in the current work were of analytical grade.

2.2. Recombinant WSP (rWSP) production by cloning, expression, and purification

rWSP production was required for examining their impact on ROS level in HepG2 cells exposed to EtOH.

The WSP of *Wolbachia* from *E. sorbillans* was produced in recombinant form by cloning, expression, and purification as stated in our previous articles [34,35] which is elucidated below.

The gene encrypting for the WSP, procured from genomic DNA withdrawn from *E. sorbillans*, was amplified by means of polymerase chain reaction (PCR) with the help of primers. The sequence of a forward primer used was 5′-CGA ATT CAT ATG GAT CCT GTT GGT CCA ATA AGT G-3′ and the sequence of a reverse primer used was 5′-GCC TCG AGT CTA GAC CTA GAA ATT AAA CGC TAC-3′. The forward primer was attached with the NdeI restriction enzyme site and the reverse primer was attached with the XhoI restriction enzyme site. The amplified PCR product (WSP gene) thus obtained was cloned into the pET 19b Expression vector. The pET 19b-WSP plasmid was transposed into the competent cells of *Escherichia coli* DH5-α. The transposed clones were investigated for the presence of WSP gene by way of Electrophoretic mobility evaluation. Lagging in electrophoretic mobility was observed in the clones having the WSP gene when compared to that of the clones without WSP gene (data not given). Lagging noticed in the electrophoretic mobility of the clones verified the presence of WSP gene. The presence of the WSP gene in the lagged clones was further investigated and verified by PCR analysis depending on the amplification of the WSP gene with the help of primers. Amplification of the WSP gene using primers verified the presence of WSP gene in the lagged clones (data not given). Clones verified for the presence of WSP gene was shifted into *E. coli* BL21 (DE3) and the expression of the His-Tagged recombinant WSP (His-Tagged rWSP) was induced by 1 mM

Isopropyl β-D-1-thiogalactopyranoside (IPTG). After induction, incubation of cells was done for 4 hours in 37°C, afterwards 1.5 ml culture was taken and centrifugation was carried out for 5 minutes at 6,000 rpm. The cell pellet was obtained and the same was suspended in 50 µl of 1× TE and 5 µl of Cell Lysis buffer (have 40 mg/ml Lysozyme supplemented by 800 U/ml DNase and 24 U/ml RNase) was put. This was incubated for duration of 30–60 minutes in 37°C and centrifugation was carried out for 5 minutes at 8,000 rpm. For the pellet (insoluble protein) got, 6 M Urea was put. This induced and lysed culture, that is clones were investigated for the expression by using 12% SDS PAGE (Sodium Dodecyl Sulfate Polyacryl Amide Gel Electrophoresis) gel and executing Sodium Dodecyl Sulfate Polyacryl Amide Gel Electrophoresis for 4 hours at 100 V continued by staining and destaining (data not given). Clones that had excellent expression was made use for further procedure of Purification and Dialysis.

The recombinant His-tagged WSP expressed was purified by way of immobilized metal affinity chromatography (IMAC) using Nickel affinity absorption chromatography that is nickel-nitrilotriacetic acid (Ni-NTA) column. The cell pellet having the recombinant protein was solubilized by way of sonication with 6 M Urea with sonication conditions of 30% ampt and 40°C till the optical density (O.D) of cells achieves 1/10th at 600 nm Absorbance. The soluble proteins were differentiated by way of centrifugation for 20 minutes at 15,000 rpm at 4°C and the supernatant was used for purification of the recombinant protein. Supernatant possessing the recombinant protein was purified by using Ni-NTA affinity column. Ni-NTA column was equilibrated using Cell lysis buffer with 6 M Urea. Then the supernatant (soluble protein) was loaded onto the pre-equilibrated column. Unbound protein was washed using the wash buffer containing 10 mM Imidazole and the bound protein was eluted by a step elution of Imidazole (an imidazole gradient) (100 mM-300 mM Imidazole) in the Cell lysis buffer with 6 M Urea. The protein thus eluted is the purified protein. The purified protein was later dialyzed against 1× PBS buffer with 4 M Urea at 4°C for 2 hours by means of the dialysis membrane. Buffer was changed with new 1× PBS with 2 M Urea and dialyzed one more time for 2 hours at 4°C. Last dialysis was performed with buffer 1× PBS for 2 hours at 4°C. After dialysis, the dialyzed protein was investigated and verified by SDS PAGE (data not given). The dialyzed protein (*Wolbachia* Surface Protein) was transposed to a bottle and was put in storage at 4°C till further utilization.

The WSP was therefore produced by way of cloning, expression, and purification (data not given).

The recombinant WSP therefore produced was later used to examine their impact on ROS level in HepG2 cells exposed to EtOH.

2.3. Cell line and cell culture

HepG2 cell line was used in the current study. HepG2 cells were got from the National Centre for Cell Science (NCCS) Pune, India. HepG2 cells were grown and maintained in cell culture flasks having DMEM supplemented with 10% FBS, Penicillin (100 Units/ml), Streptomycin (100 µg/ml) and 2 mM L-glutamine by incubating in 5% CO_2 at 37°C in a CO_2 incubator.

2.4. Treatment for cells

Cells were cultured until they became confluent. After reaching 75%–80% confluence, the cells were treated with 56.57 mM EtOH alone, 56.57 mM EtOH + 150 µg/ml WSP, and 56.57 mM EtOH + 250 µM Silymarin (SIL) for 24 hours. Initial experiments were performed to choose the concentration and time of exposure of EtOH, WSP, and SIL based on the cell viability analysis by MTT assay (data not given). After incubation, the cells were used for the assay described beneath.

2.5. Assessment of ROS level in HepG2 cells by Flow cytometry using H2DCFDA staining assay

ROS level was measured in HepG2 cells by means of FACSCalibur Flow cytometer (BD Biosciences, San Jose, CA) using ROS-sensitive, oxidation sensitive, redox-sensitive, cell-permeable non-fluorescent dye, H2DCFDA/DCFH-DA [17,29,36,37]. H2DCFDA/DCFH-DA is a broadly utilized ROS indicator to detect ROS. H2DCFDA-based evaluation is the most general procedure for directly quantifying the redox state of a cell. It has a number of benefits like it is highly easy to utilize, very sensitive to changes in the redox condition of a cell, low-priced and may be utilized to follow changes in ROS over time [15,16, 36]. ROS was determined with modification to the protocol as described in the Chang et al. [16] Research article.

H2DCFDA staining of cells determines intracellular ROS level on the basis that H2DCFDA/DCFH-DA, a lipid-soluble, membrane permeable, primarily non-fluorescent compound get cleaved by intracellular non-specific esterases to eliminate the diacetate part and form the membrane-impermeable non-fluorescent product, H2DCF. H2DCF is oxidized by intracellular ROS to generate a highly fluorescent compound DCF which is a measure of the quantity of ROS present in the cells that emits green fluorescence [15,18,19,28–32]. The DCF fluorescence which indicates intracellular ROS level [18,19,28] was then determined in H2DCFDA stained HepG2 cells by flow cytometer.

To determine the ROS level, HepG2 cells were grown in a 6-well plate at a density of 3×10^5 cells/2 ml and were incubated for 24 hours at 37°C in 5% CO_2 incubator. The spent medium was sucked out and washed using 1 ml 1× PBS. Later the cells were incubated with and without 56.57 mM EtOH individually for 24 hours and were afterwards treated with 150 µg/ml WSP and 250 µM SIL individually for 24 hours. Following WSP or SIL treatment, the medium was taken out from all the wells, shifted to the tubes and washed using 500 µl PBS (the PBS was stored in the same tubes). Cells remained Untreated (UT) were utilized as control. PBS was removed, 180 µl of Trypsin-EDTA solution was added and incubated at 37°C for 3–4 minutes. Culture medium was transferred back into their respective wells, the cells were picked and transferred into the tubes. The tubes containing the cells were centrifuged for 5 minutes at 300 × g at 25°C. The supernatant was poured out carefully and the pellet was washed twice with PBS. The PBS was decanted completely. 0.5 ml 2% Paraformaldehyde solution was added and kept to incubation for 20 minutes. Then, washed by using 0.5% Bovine serum albumin (BSA) in 1× PBS. 0.1% Triton-X 100 in 0.5% BSA solution was added, kept to incubation for 10 minutes and then washed by using

0.5% BSA in 1× PBS. The H2DCFDA stock solution (4 mM) was diluted using DPBS to prepare 10 µM working solution. Cells were incubated with 10 µM H2DCFDA at a density of 1 × 10^6 cells/ml at 37°C in the dark for 30 minutes. Cells were washed by centrifugation at 150 × g for 5 minutes. Then, the supernatant was discarded and the cells were resuspended smoothly in 400 µl pre-warmed DPBS. ROS level in the cells was evaluated by FACSCalibur flow cytometer. DCF fluorescence shows the ROS level in cells [18,19,28]. The percentage of ROS level in cells, stained by H2DCFDA was evaluated in each one of the samples.

2.6. Flow cytometry analysis of ROS data

Flow cytometric (FACS) analysis of the quantity of ROS generated in the HepG2 cells was determined by using H2DCFDA stain by means of a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Data collection was made and examined by using FACSCalibur flow cytometer having a 15 mW, 488 nm air-cooled argon-ion laser and the software CellQuest Pro (BD Biosciences, San Jose, CA). To evade debris, gating was done for the population of the cells to be analyzed using forward scatter (FSC) and side scatter (SSC). Cells were evaluated in each one of the samples by means of a flow cytometer [18,28].

ROS was measured by H2DCFDA staining. DCF, which is a measure of the quantity of ROS present in the cells [18,19,28] that emits green fluorescence [30–32], was measured by FACSCalibur flow cytometer using the 488 nm laser for excitation and by filtration via 535 nm emission filter. The DCF fluorescence intensity was read on the FL1 channel for the cells labeled with H2DCFDA by FACSCalibur flow cytometer [18,28]. A dot plot was created to evaluate the size and granularity of the cells. In the dot plot, the *X*-axis represents the FSC parameter which is relative to the size of the cell and the *Y*-axis shows the SSC parameter which correlates with the components of the cell. Histograms were evaluated for H2DCFDA stained cells. Histogram of H2DCFDA shows the intensity of DCF of cells in the FL-1 channel and the cell count of DCF fluorescence. In the histogram of H2DCFDA, the *X*-axis represents the DCF fluorescence intensity, while the *Y*-axis indicates the number of cells with DCF fluorescence. DCF fluorescence shows the ROS level in the cells [18,19,28]. The level of DCF fluorescence intensity in the cells was determined by using FACSCalibur flow cytometer.

2.7. Statistical analysis of data

Statistical analysis was made by the statistical software, GraphPad Prism 6.0 Software (GraphPad Software, Inc., San Diego, CA). Data were shown as the mean ± Standard Deviation (SD) of three independent experiments done. The data were statistically examined by means of Analysis of Variance (ANOVA) proceeded by means of Tukey's test. *p*-values less than 0.05 ($p < 0.05$) were regarded to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Evaluation of impact of WSP on ROS level in EtOH-exposed HepG2 cells by flow cytometry

In the present study, we determined the impact of WSP on ROS level in HepG2 cells by incubating with and without 56.57 mM EtOH independently for a period of 24 hours and then treating the cells with 150 µg/ml WSP and 250 µM SIL independently for a period of 24 hours. Later the cells were stained using H2DCFDA dye and ROS in the cells were quantified by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Cells were taken up for evaluation by CellQuest Pro software (BD Biosciences, San Jose, CA) and measurement of ROS level in the cells, as the percentage of the entire cell population, was carried out. We measured the ROS level in cells using FACSCalibur flow cytometer through the identification of DCF fluorescence. DCF fluorescence indicated the ROS level in the cells.

We detected that the ROS level was less in UT HepG2 cells. The ROS level was elevated in HepG2 cells after 24 hours of exposure to EtOH. WSP treatment for a period of 24 hours lessened the ROS level in EtOH exposed HepG2 cells and also SIL treatment for a period of 24 hours lessened the ROS level in EtOH exposed HepG2 cells as evaluated by H2DCFDA staining by means of flow cytometer (Figs. 1–4) (Table 1) (Fig. 5).

Flow cytometer analysis of H2DCFDA staining showed that EtOH exposed HepG2 cells treated with WSP lessened the increased ROS level and oxidative stress. Therefore, this H2DCFDA staining assay results propose that WSP has a protecting impact against the EtOH-induced increase in ROS level and oxidative stress in HepG2 cells. The observations of the current investigation propose that WSP could serve to be a cytoprotective agent.

Previous studies showed that the long-term excessive intake of alcohol leads to the development of the alcohol-related disease, which is the reason for death among all liver diseases [22,38]. Oxidative stress is regarded as one of the important processes

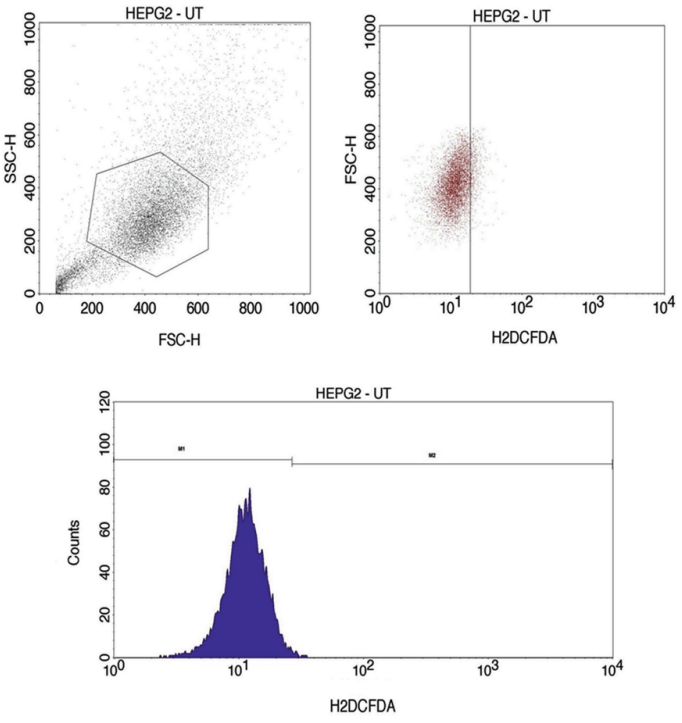

Figure 1: Typical illustration of Flow cytometric study findings of ROS level in the UT HepG2 cells. Flow cytometric study findings indicated are the typical data of three separate experiments performed.

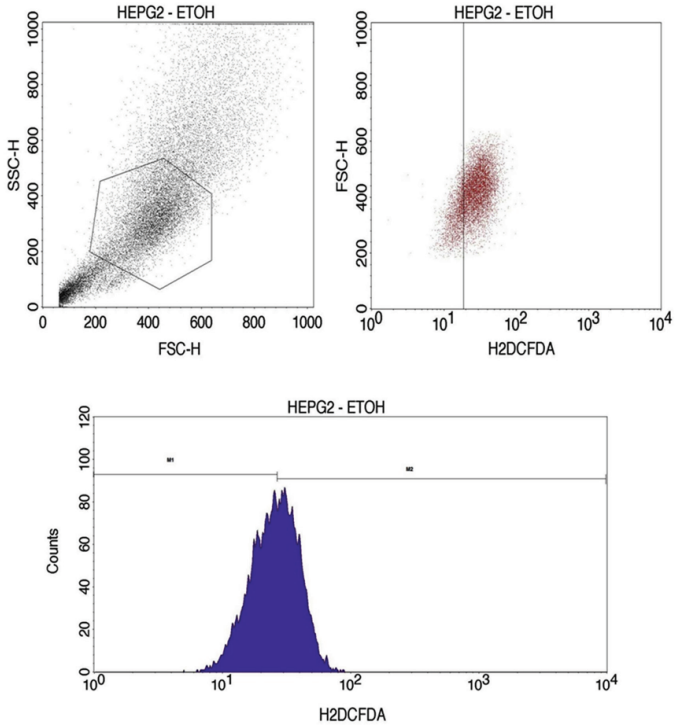

Figure 2: Typical illustration of Flow cytometric study findings of ROS level in the HepG2 cells incubated with EtOH for 24 hours. Flow cytometric study findings indicated are the typical data of three separate experiments performed.

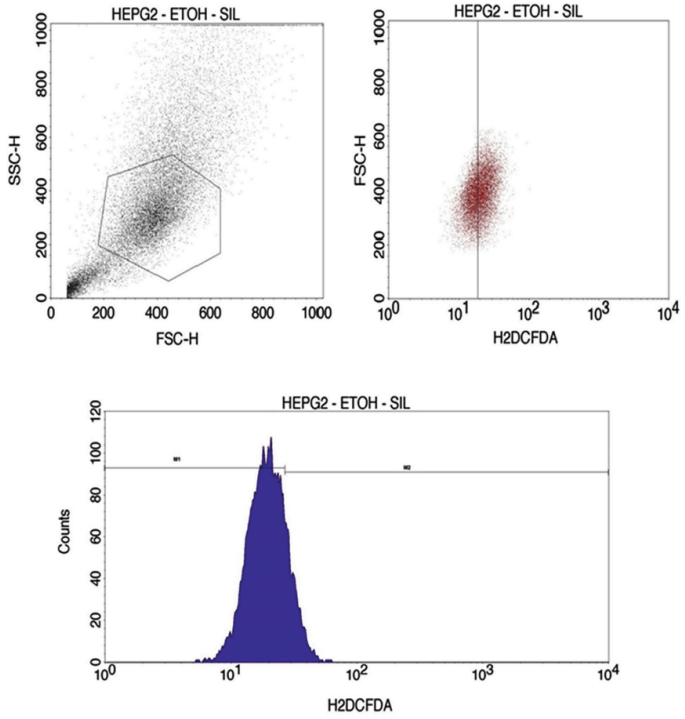

Figure 4: Typical illustration of Flow cytometric study findings of ROS level after SIL treatment for 24 hours to HepG2 cells incubated with EtOH. Flow cytometric study findings indicated are the typical data of three separate experiments performed.

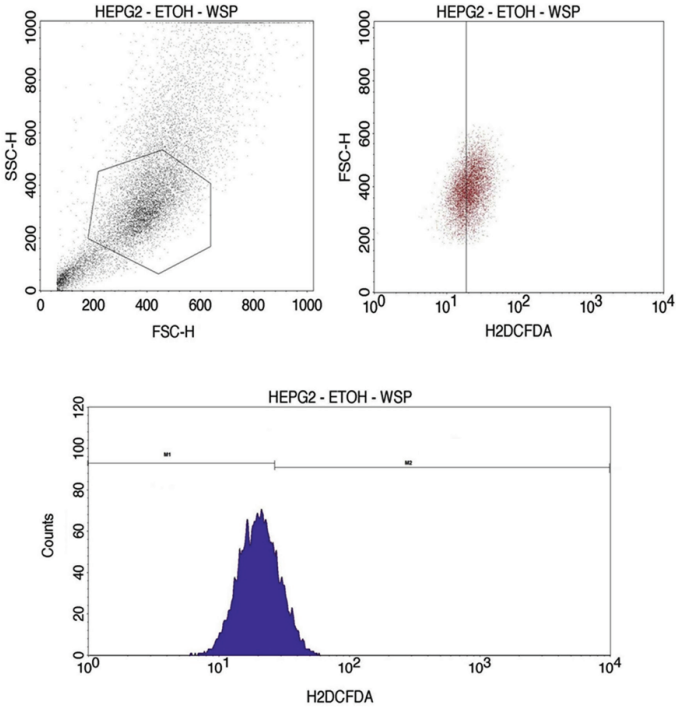

Figure 3: Typical illustration of Flow cytometric study findings of ROS level after WSP treatment for 24 hours to HepG2 cells incubated with EtOH. Flow cytometric study findings indicated are the typical data of three separate experiments performed.

responsible for alcoholic liver diseases which include alcoholic fatty liver, alcoholic hepatitis, fibrosis, cirrhosis, or cancer [22,38]. Oxidative stress is described as an enhancement in ROS and/or a reduction in the antioxidant protection process [7]. EtOH is identified to induce Cytochrome P450 2E1 (CYP2E1) which results in the enhanced production of ROS. CYP2E1 is identified as an effective producer of EtOH-mediated ROS production, leading to apoptotic cell death [22,38]. The enhancement of EtOH-induced CYP2E1 action is recommended to be an important provider in producing a condition of oxidative stress that leads to hepatotoxicity. The increased level of ROS finally mediates hepatic tissue damage caused by EtOH [38]. In the metabolism of EtOH to acetaldehyde in the body, a condition of oxidative stress is produced by more ROS production, which plays an important function in the development of alcoholic liver disease/EtOH-mediated liver tissue injury [22,38].

Earlier study disclosed that WSP impedes apoptosis in human polymorphonuclear cells (PMNs) [2]. However, there was no experimental data about finding out the protecting impact of WSP (of *Wolbachia* of Uzifly) against the EtOH-induced increase in ROS generation and oxidative stress in HepG2 cells by means of flow cytometry. The present study showed that WSP might render a protecting impact against the EtOH-induced increase in ROS generation and oxidative stress in HepG2 cells. WSP has an alike impact as that of SIL, which has hepatoprotective and

Table 1: Data for percentage of ROS level in HepG2 cells.

Groups	Percentage of ROS level
UT	0.8933 ± 0.07506
EtOH	49.72 ± 1.00
EtOH-WSP	23.06 ± 0.6580
EtOH-SIL	19.33 ± 1.354

Data showed are the mean ± SD of three separate experiments (n = 3) performed.

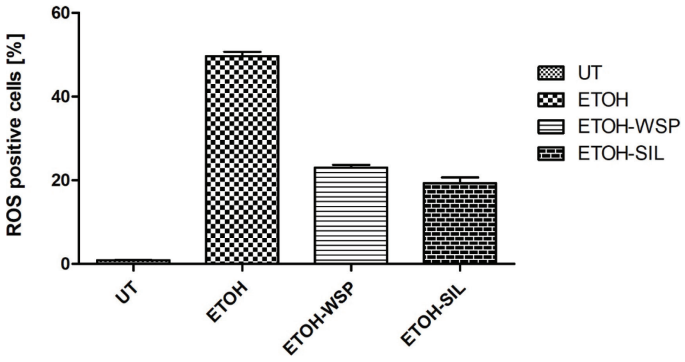

Figure 5: Graphical picture of data for percentage of ROS level in HepG2 cells. Data marked in the bar graph indicates the mean ± SD of three separate experiments (n = 3) performed. Significance: EtOH was observed to be highly significant compared to UT with a p-value of $p < 0.0001$. EtOH-WSP and EtOH-SIL were observed to be highly significant compared to EtOH with the p-value of $p < 0.0001$.

antioxidative properties [33]. Thus, the current study proposes that WSP could be used as a powerful therapeutic drug to treat EtOH-related liver ailments.

4. CONCLUSION

The current study disclosed that WSP demonstrates cytoprotective impact against ROS enhancement induced using EtOH in HepG2 cells by lessening the ROS. Thus, the current study proposes that WSP could be used as a powerful therapeutic drug to treat EtOH-related liver ailments.

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