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Serum deprivation enhanced ethanol-induced toxic responses in A549, lung carcinoma cells

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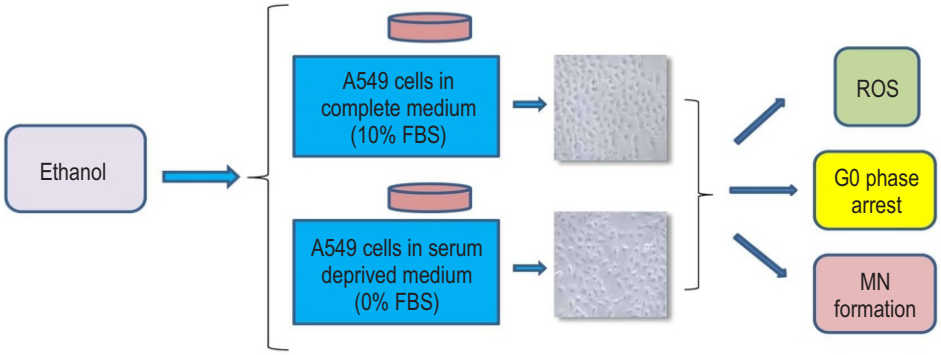
Abstract

Aim: The current study explores the toxic consequences of ethanol on human lung carcinoma cell, A549 in serum-deprived condition.

Methodology: Human lung carcinoma cells, A549, were cultured in complete and serum-deprived medium for 6 hr. Subsequently, they were exposed to 50 mM and 100 mM concentrations of ethanol. Cytotoxicity studies linked with cell viability, oxidative stress, cell cycle arrest and micronuclei formation were performed using various toxicological parameters, namely MTT assay, DCFDA based ROS generation, cell cycle analysis and micronuclei formation assay. The cytotoxicity of ethanol in complete and serum deprived medium were compared at similar doses and time duration.



Results: The metabolic viability assay demonstrated that 50 mM and 100 mM concentration of ethanol did not induce significant levels of cytotoxic alteration in A549 lung carcinoma cells in complete medium. However, in serum-deprived conditions, 50 mM and 100 mM ethanol concentration significantly altered cell viability. Further, exposure of 50 mM and 100 mM concentration of ethanol enhanced reactive oxygen species levels in A549 cells more significantly in serum-deprived conditions than in complete medium. In addition to cytotoxicity and oxidative stress, 50 mM and 100 mM ethanol also arrested the cells at G0 phase more significantly in serum deprived conditions compared to complete medium.

Interpretation: Both 50 mM and 100 mM ethanol concentration enhanced the cell cytotoxicity and reactive oxygen species, cell cycle arrest and micronuclei formation more severely in serum-deprived medium than in complete medium (containing 10% FBS) under similar treatment conditions.

Key words: A549 cells, Cytotoxicity, Ethanol, Micronuclei formation, Serum deprivation

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Introduction

Globally, cancer cases have been estimated to rise to 19.3 million and approximately 10 million deaths in the year 2020, as per the reports of GLOBOCAN 2020 generated by the International Agency for Research on Cancer (Sung *et al.,* 2021). Similarly, as per the Cancer Statistics Report- 2020 of India produced by the National Cancer Registry Programme, India the estimated number of cancer patients in India was 1,392,179 for the year 2020 (Mathur *et al.,* 2020). The term cancer incorporates multiple variants. Among them, the seriousness of lung cancer has increased multifold in the recent past. Lung cancer is the largest cause of cancer death despite advancements in our understanding of risk, progression, immunological control, and therapeutic choices (Bade and Dela Cruz, 2020). According to the above report, there were 2.09 million cases reported and 1.76 million deaths in 2018 due to lung cancer.

The cancer statistics of India delineate 6.9 percent of lung cancer cases. Although there are multiple riskfactors of lung cancer. These incorporate smoking tobacco, exposure to radon and other hazardous chemicals, petroleum products (Field and Withers, 2012). Ethanol or ethyl alcohol is an organic compound and world widely utilized for consumption as alcoholic beverages. Several research investigations have established that regular alcohol consumption is hazardous (Rehm, 2011; Iranpour and Nakhaee, 2019). It incorporates mental and physical illness. Alcohol consumption is related to enhanced incidence of neoplasms. Alcohol abuse encompasses certain cancers, namely squamous cell carcinoma of oral cavity, hepatocellular, pancreatic, breast, and oropharyngeal cancer (Johnson, 2020). Alcohol is also one of the suspected risk factors in lung cancer progression (Boffetta and Hashibe, 2006; Zhou *et al*., 2016; Seitz and Stickel, 2007; Troche *et al*., 2016).

International Agency for Research (IARC) on Cancer has categorized alcohol under Group 1 carcinogen. However, the relation between alcohol intake and increment in lung cancer chance has been also well documented (Bagnardi *et al.,* 2010). The epidemiological studies have reported that alcohol-based deaths have increased significantly in recent years and has become the prominent basis of disability (Iranpour and Nakhaee, 2019). The plethora of studies on several cell lines and animal models are available which has well established the function of ethanol in inducing cell death via apoptosis (von Haefen *et al*.,2011; Oliveirada-Silva *et al*., 2009; Arzumanyan *et al*., 2009). The reports suggest that alcohol induces oxidative stress at cellular level in a dose-dependent manner (Das and Vasudevan, 2007). The multiple fates such as inhibition of mitogenic growth factor signalling, glutathione depletion, DNA damage, and endoplasmic reticulum stress are well recognized via which ethanol promotes cellular apoptosis (Plante *et al.,* 2013).

Although liver metabolizes alcohol, ethanol intake might impact liver and other organs (Akbar *et al.,* 2018; Chernyavsky *et al.,* 2020; Bishehsari *et al.,* 2017). Recent past studies also suggested that alcohol can significantly increase the risk of various pulmonary settings such as alcoholic pneumonia, lung disease, acute respiratory distress syndrome (ARDS) and acute lung injury (Kaphalia and Calhoun, 2013; Kato *et al*., 2005). In the present study, we have taken A549 cell lines, a well-established in vitro model system for cytotoxicity analysis (Relja *et al*., 2016; Mathew *et al*., 2015). Inadequacy in nutrition can also lead to various health issues (Ruel *et al.,* 2013).

Among numerous environmental factors governing the pattern of cancers, nutrition is one of the key factors influencing the risk at the cellular and molecular level (Wiseman, 2019). Despite many epidemiological data, in-vivo studies and in-vitro investigations are available, which conclude the cytotoxic nature of ethanol (Nurmi *et al*., 2009). However, studies on the cytotoxic effects of ethanol under serum-deprived conditions using lung carcinoma is sparse. Thus, our present study was performed to examine the effects of ethanol exposure in A549 cells under serum deprived conditions and complete medium.

Materials and Methods

Cell culture condition: Human lung adenocarcinoma (A549) was utilized as in-vitro model system. Initially, the cell line was acquired from cell repository of National Centre for Cell Science, Pune, (India) and further sustained as per the established procedures at Central Cell Culture Facility, Council of Scientific and Industrial Research- Indian Institute of Toxicology Research, Lucknow, India. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. Cells were incubated at 37°C in a CO_2 incubator.

Established A549 model system: A549 cells were cultured in DMEM supplemented with 10% FBS and underwent overnight incubation for 10-12 hr. Further, the medium was replaced with DMEM not supplemented with FBS (serum deficient medium) and incubated for 6 hr. Post incubation, A549 cells were exposed to different doses of ethanol (50 mM till 400 mM) for 24 hr. Also, A549 cells were cultered in complete medium and treated with 50 mM till 400 mM concentration of ethanol for 24 hr. Post treatment, the impact of ethanol was examined in complete and serum-depleted media. Hydrogen peroxide under similar conditions served as a positive control.

MTT Assay: MTT 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) assay was conducted according to the procedure of Khan *et al.* (2019) with slight change. In short, A549 cells ($1x10^4$) were cultured in 96 well culture plates and then incubated at 37°C for 24 hr. Following that, the cells were exposed to ethanol at various doses (50, 100, 200, and 400 mM) along with a positive control H_2O_2 (100 µM). The IC_{50} values (the half-maximal inhibitory concentration) of ethanol is approximately 500 mM. Later, the treated medium has been removed and washed with 1X PBS and each well was

supplemented with culture medium containing 5 mg ml^{-1} MTT dye for 4 hr. After incubation, the medium was withdrawn and each well was treated with 200 µl of dimethyl sulphoxide. The absorbance was read using a multi-well microplate reader at 550 nm.

Determination of morphological alterations: Morphological alterations due to ethanol exposure (50 mM and 100 mM for 24 hr) in A549 cells in complete (DMEM with 10% FBS) and in serum-deprived condition (DMEM without FBS) had been observed at 20X magnification utilizing a phase contrast microscope.

ROS generation assay: The generation of reactive oxygen species by ethanol was monitored using a flow cytometry assay based on 2', 7'–dichlorofluorescin diacetate following the method of Kumar *et al.* (2015) with slight changes. Briefly, A549 cells ($1x10^5$ cells per well) were cultured in complete as well as in serum-deprived condition (DMEM without FBS). These cell lines were exposed to different concentrations of ethanol (50 mM and 100 mM) for 6 hr. Warm trypsin was used to collect the exposed cells, which were then washed with 1X PBS and resuspended in DCFDA solution. The fluorescent intensity of incubated cells (20 min at 37°C temperature and 5% CO_2 in a incubator) was analysed by flow cytometer (BD FACS Canto ™ II, USA).

Cell cycle analysis: Cell cycle was studied via flow cytometry using a DNA-binding stain-PI (Singh *et al*, 2017). Briefly, A549 cells cultured ($1x10^5$ cells per well) under complete media condition (DMEM with 10% FBS) and serum-deprived media condition. For 24 hr, the cells were treated with ethanol at 50 and 100 mM doses.The cells were trypsinized and washed using 1X PBS (twice), then fixed with 70% chilled ethanol. The ice-cold ethanol was added to pellet while vortexing. Following an overnight incubation period at 4°C, the cells were collected by centrifugation at 2000 rpm for 5-10 min. The collected cells were resuspended in 1X PBS and treated with RNase A (500 U ml^{-1}) to degrade excess RNAs. Propidium iodide was used to stain cell pellets for 30 min at 4 °C in dark. The cell cycle was assessed using a flow cytometer BD FACS Influx, USA. The software (BD FACS™ Software 1.2.0.87) was used for cell cycle analysis.

Flow cytometry-based micronucleus assay: The flow cytometric micronucleus assay (FMN) was performed as per the standard protocol of Pandey *et al*. (2009). A549 cells cultured under complete media (DMEM with 10% FBS) as well as in serum-deprived condition (DMEM without FBS) were exposed to different doses of ethanol (Eth-50 mM and Eth-100 mM) for 24 h. Post-treatment replaced the medium with a complete medium (DMEM with 10% FBS) and incubated A549 cells for 48 h (at least

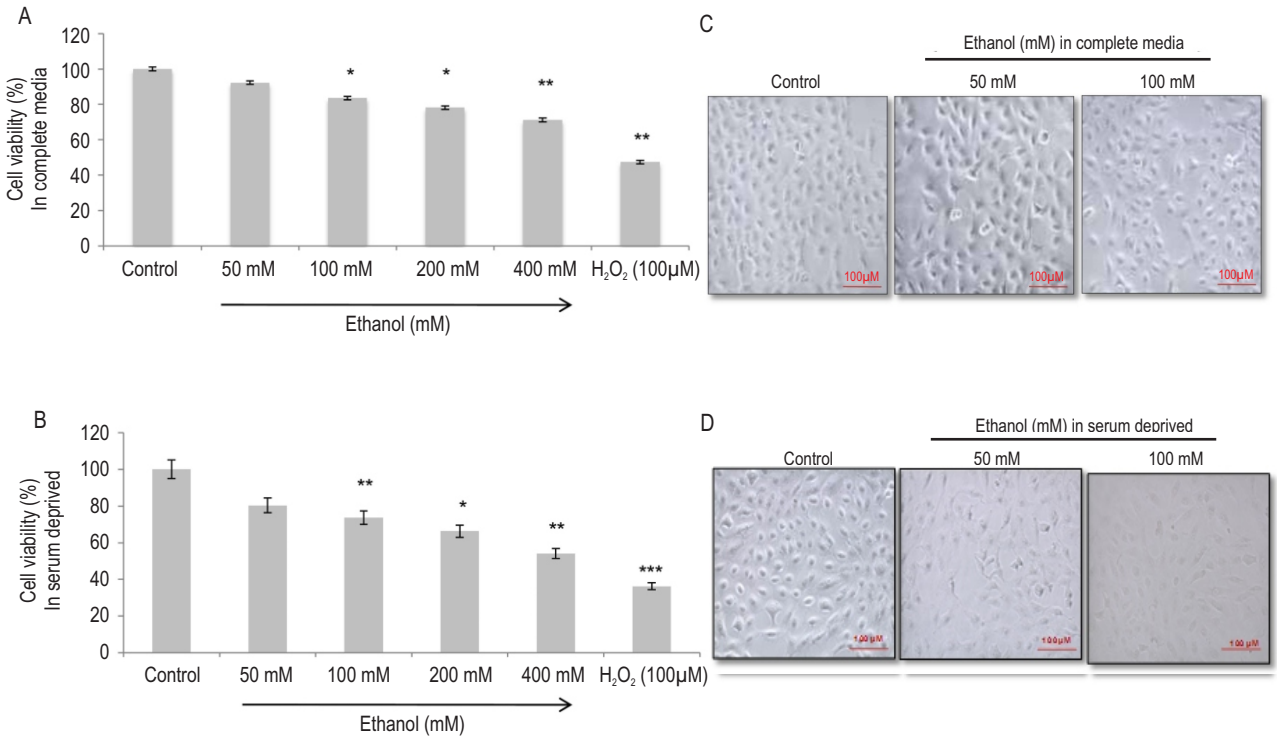

Fig.1: Ethanol's effect on the morphology and viability of A549 cells. (A) and (B) MTT test was used to determine the percent cell viablity in A549 cells exposed to various doses of ethanol for 24 hours in complete and serum-deprived media.(C) & (D) Representative phase-contrast images (20x objective) show morphological changes in A549 cells cultured in complete medium and serum-deprived medium and exposed to ethanol. All data expressed as mean ± SD (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ with respect to their control).

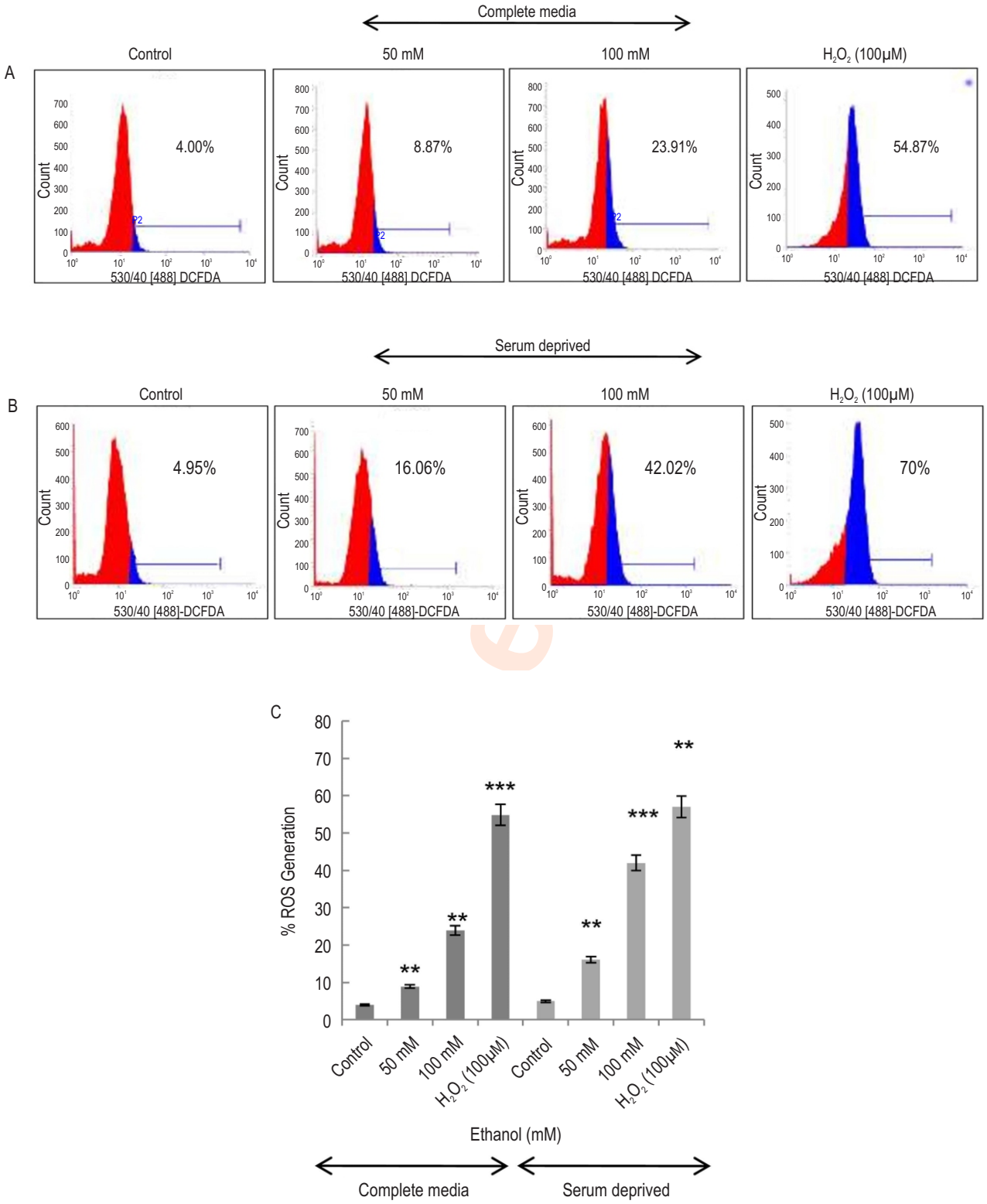

Fig. 2: Quantification of ROS induced by ethanol via flow cytometry (A & B) Flow cytometry analysis (by using DCFDA dye) and (C) Bar graph of ROS generation in A549 cells cultured in complete medium (CM) and also in serum-deprived condition (SD) exposed to different concentrations of ethanol (50 mM and 100 mM) for 6 h. All results are shown as mean ± standard deviation (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ in comparison to their control).

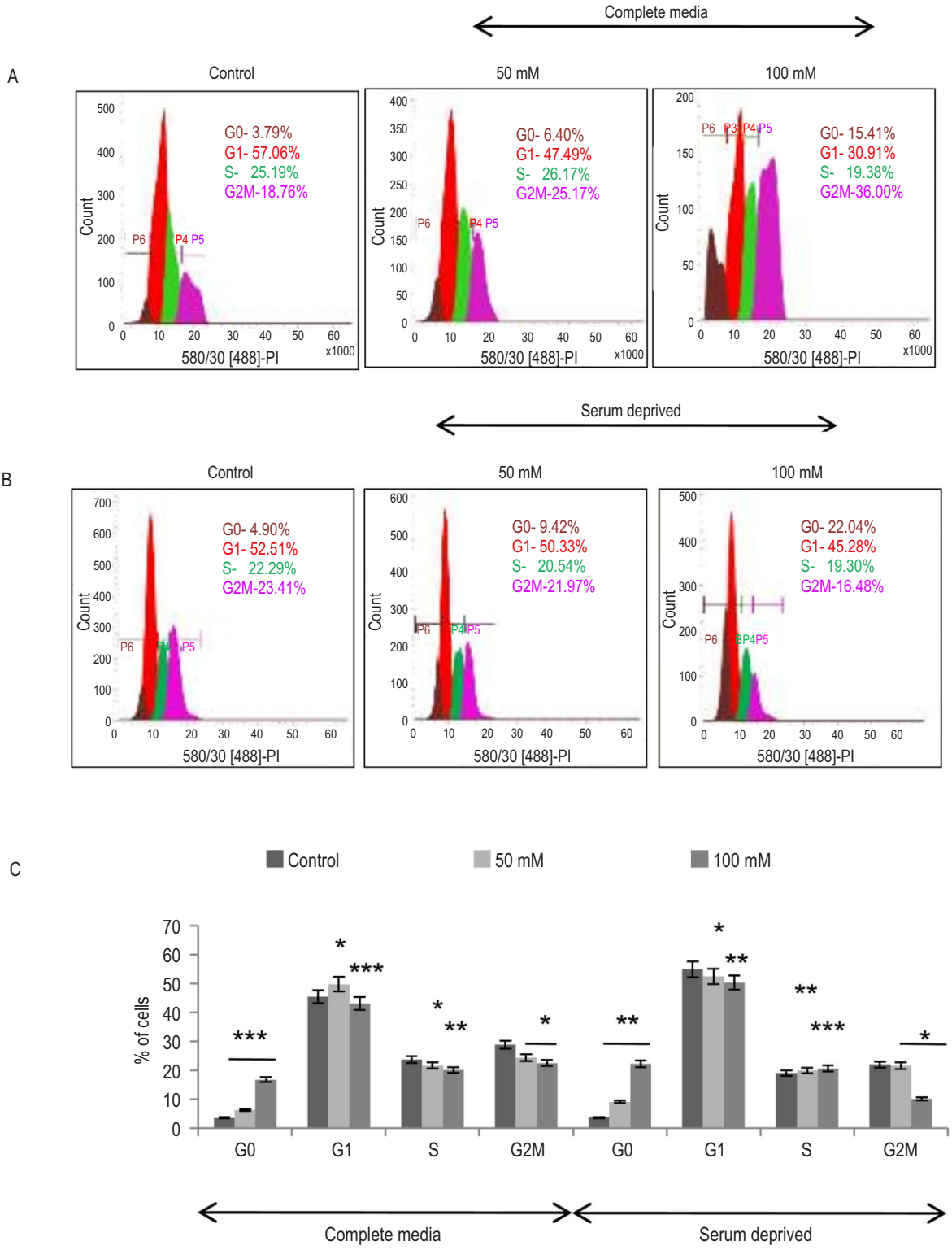

Fig. 3: Consequences of Ethanol on cell cycle activity (A & B) Evaluation of the effect of different concentrations of ethanol viz. 50 mM, 100 mM exposed to both A549 cells cultured in complete medium, *i.e.,* 10% FBS and serum-deprived condition (SD), *i.e.,* without serum. The fluorescence of propidium iodide (PI) is detected using flow Cytometry. Propidium iodide, a stoichiometric dye, *i.e.,* bind in proportion to the amount of DNA present in the cell (C) Bar graph of alterations in cell cycle phases. All data expressed as mean ± SD (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ with respect to their control).

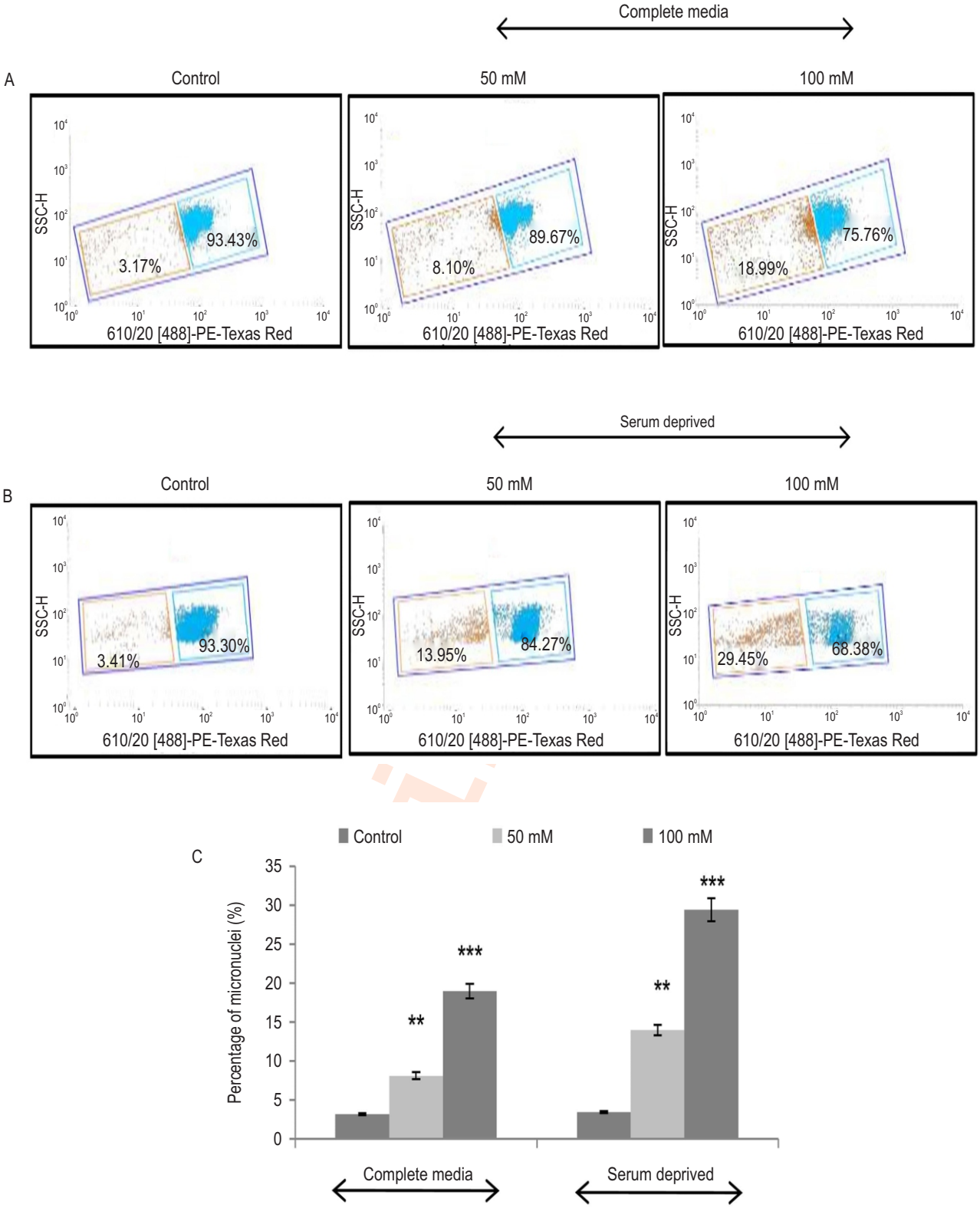

Fig. 4: Ethanol's effect on the genomic instability of A549 cells (A & B) micronuclei assay by flow Cytometry (FMN) and (C) Bar graph of micronuclei assay in A549 cells cultured in complete media (CM) and in serum deprived medium (SD-without FBS), exposed against different concentrations of ethanol 50 mM, 100 mM for 24 hrs. (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ with respect to their control).

two cell cycles). The cells were collected after trypsinization and centrifugation. Harvested cells were resuspended in 1 ml of Solution I (containing 10mM NaCl, 3.4mM sodium citrate, 10 mg l^{-1} RNAse, 0.3 mg l^{-1} igepal, 25 mg l^{-1} EtBr), vortexed, and incubated at room temperature for 1 h. 1 ml of Solution II (1.5% citric acid, 0.25M sucrose, 40 mg l^{-1} EtBr) was added and mixed. After 15 min, the suspension was kept at 4 ºC until analysis.The flow cytometry micronuclei assay was carried out using a blue laser (488 nm) and a 610 nm bandpass filter of FACSTM Influx flow cytometer, BD Biosciences, USA. A total of 10,000 events were acquired. The software BD FACSTM Software 1.2.0.87 has been utilized for acquisition and analysis.

Reagents and Chemicals: The required high-grade chemicals were procured from Sigma-Aldrich chemical company Pvt. Ltd., USA. The fluorescent dyes were purchased from BD Bioscience, USA. Ethanol was purchased from Merck Millipore, Germany. The other notable media of GIBCO™ brand DMEM, antibiotics-antimycotic solution (100X), FBS were procured from Thermo Fisher Scientific, USA. The high-grade plastic lab wares were purchased from Tarsons Products Ltd., Kolkata, India; similarly, finest quality laboratory glasswares were procured from Borosil, Mumbai, India.

Statistical analysis: The result interpretation was presented as mean +/- standard deviation of the data. The experimental data were analyzed systemically using one-way ANOVA in Microsoft Excel 2007. The result figure produced indicates that *p <0.05 is considered to be significant, **p <0.01 is regarded more significant, and ***p <0.001 is considered extremely significant.

Results and Discussion

The operational idea of the present investigation involved the study of cytotoxic impacts of ethanol on representative human lung adenocarcinoma (A549 cell line) under serum-deprived conditions. The study was also carried to confirm whether ethanol exposure in serum-deprived condition had any impact compared to the cells exposed to the same concentrations of ethanol in cells cultured in a complete medium. In the present study, we observed a decreased cell viability of A549 cells in a concentration-dependent manner of ethanol treatment for 24 hr. Exposure to 50 mM ethanol did not elicit any cytotoxic response. However, a significant reduction in the percentage of viable cells was found at a dose of 100 mM ($p < 0.1$). (Fig. 1A, B). The results of ROS generation acquired by flow cytometric-based DCFDA assessment are shown in Fig. 2A; B. A549 cells cultured in complete medium and serum-deprived medium were exposed to 50 mM and 100 mM. Moreover, a significant increase in ROS generation in serum-deprived conditions was shown at 50 mM ($p < 0.01$) and 100 mM ($p < 0.001$) than in the complete medium and control (positive control H2O2). Our result, consistent with previous studies, ethanol induces cellular ROS production, which may be the primary cause of adverse cytotoxic effects (Das and Vasudevan, 2007; Flora *et al*., 2012; Olney *et al*., 2000).

Cellular oxidative stress is a turnout of divergence between ROS and the antioxidant protection system (Birben *et al.,* 2012). The range of cellular abnormalities results from oxidative stress damaging DNA and altered protein translation. The results of cell cycle analysis are presented in Fig. 3A& B, ethanol-induced DNA damage via ROS in A549 cells and arrested the A549 cells more significantly in the serum-deprived medium compared to complete medium and untreated control for 24 hr. Furthermore, cell cycle arrest in the G0 is dose-dependent, 50 mM (9.42%) and 100 mM (22.04%) of ethanol-mediated toxicity. Similarly, the data demonstrated an increase in the G2M cell cycle phase in A549 cells exposed to 50 mM and 100 mM ethanol compared to the untreated control. The possible mechanism is that the cell cycle arrest must persist for the length of the damage, and it is also possible that the activity of cyclin/CDKs is altered during the arrest. Cell cycle re entry should occur only from a state of cyclin activation that ensures the proper sequence of DNA replication and mitosis (Toettcher *et al*., 2009)

The previous studies established that ethanol inducing cell death (von Haefen *et al*.,2011; Oliveirada-Silva *et al*., 2009; Arzumanyan *et al*., 2009).The micronuclei formation assessment using flow cytometric data highlights are summarized in Fig. 4A, B. Micronuclei assay by flow cytometry revealed a statistically remarkable induction in DNA damage in A549 cells cultured in complete medium and exposed to different ethanol concentrations in a dose-dependent manner (Kayani and Parry, 2010). A significant increase in micro-nuclei was observed at 50 mM (8.10%) and 100 mM (18.99%) ethanol treatment in A549 cells, respectively. However, a statistically significant elevation in micro-nuclei percentage at 50 mM (13.95 %, $p < 0.01$) and 100 mM (29.45 %, $p < 0.001$) was found in ethanol exposed in serum-deprived A549 cells (Boffetta and Hashibe, 2006; Zhou et al., 2016; Seitz and Stickel, 2007; Troche *et al*., 2016) also advocated our finding.

Based on the present study, it can be accomplished that ethanol can induces, cell cycle perturbations and genotoxic effects at 50 mM and 100 mM ethanol concentration. However, our study unequivocally reflects that exposure on A549 cells under serum deprivation-induced significantly elevated ROS and higher frequencies of MN and alteration in the G0 phase of the cell cycle. The findings of the in-vitro study suggested that ethanol enhanced the cytotoxicity and micronuclei formation more significantly in the serum-deprived medium than complete medium in lung adenocarcinoma (A549) cells. The detrimental effect of ethanol consumption increased significantly under nutrient-deficient conditions. Research investigations are further required to explore the effect of ethanol on serum-deprived condition at the gene level, as the nutrient is indeed one of the environmental factors influencing gene expression.

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Add-on Information

Authors' contribution: P. Khare: Design of experiments, experimentation, result analysis, manuscript preparation ; **V.K. Singh:** Statistical analysis of data, manuscript editing; **Lakshmi Bala:** Conceptualization, supervision, overall critical analysis of MS.

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