Under lithium carbonate administration, nicotine triggers cell dysfunction in human glioblastoma U-251MG cells, which is distinct from cotinine

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Abstract. Nicotine is an alkaloid found in tobacco leaves. Smoking prevention has been a neglected issue in psychiatry; nicotine intake in conjunction with the administration of the mood stabilizer, lithium carbonate (Li_2CO_3), may negatively affect brain cells. The present study investigated the combined effects of nicotine and its metabolite, cotinine, and Li_2CO_3 compared to acetylcholine and dopamine in U-251MG human glioblastoma cells. Cell proliferation was found to be decreased by nicotine and to be further suppressed following treatment with Li_2CO_3, accompanied by mitotic catastrophe and increased levels of superoxide anion radicals. By contrast, cotinine did not exert such detrimental effects. It was also found that acetylcholine did not suppress cell proliferation, whereas dopamine in conjunction with Li_2CO_3 decreased cell proliferation in a concentration-dependent manner. The nicotine-induced cell growth inhibition was restored by mecamylamine, a non-competitive antagonist of nicotinic acetylcholine receptors. On the whole, the findings of the present study suggest that nicotine combined with Li_2CO_3 leads to the suppression of the proliferation of human glioblastoma cells accompanied by mitotic catastrophe and superoxide anion radical generation. These findings may provide further cellular biological insight into the risks associated with smoking under Li_2CO_3 administration.

Introduction

Nicotine is an alkaloid found in tobacco leaves. Nicotine stimulates the brain to secrete dopamine, temporarily clears the head, and relieves fatigue. Smoking prevalence and the associated health risks are higher in psychiatric patients. However, smoking prevention has been a neglected issue in psychiatry (1). It has been experimentally demonstrated that nicotine increases neuronal firing rates and induces the release of dopamine (2,3). In addition, nicotine metabolism occurs in the liver by cytochrome P450, and 70-80% of nicotine is metabolized into cotinine (4,5). Nicotine is rapidly removed from the central nervous system for 1-3 h, while cotinine is known to remain in the body for >20-30 h (6). There are a number of studies available on nicotine addiction (4-6); however, the effects of cotinine on the human body have not yet been fully elucidated. Lithium carbonate (Li_2CO_3) has been used as a mood stabilizer in bipolar disorder. Of note however, the effective dose range of Li_2CO_3 is 0.6-1.0 mM and >1.5 mM as lithium in serum, which is close to the addiction level of Li_2CO_3 (7). Nicotine intake in conjunction with the administration of the mood stabilizer, Li_2CO_3, may have a synergistic effect on brain cells, although this remains to be determined.

The author of the present study recently found that platinum nano colloids promoted the autoxidation of dopamine (8). The platinum nano colloids and dopamine increased intracellular reactive oxygen species generation and suppressed the proliferation of human glioblastoma cells (8). On the other hand, 2-phenylethylamine did not suppress the growth of human glioblastoma cells, regardless of the combined use with platinum nano colloids. Thus, the administration of platinum nano colloids affected the growth of human glioblastoma cells, depending on the type of coexisting neurotransmitters (8).

The present study investigated the effects of nicotine and its metabolite, cotinine, in conjunction with the use of Li_2CO_3, on the proliferation of U-251MG human glioblastoma cells, and compared these to those of acetylcholine and dopamine. The U-251MG cell line was used as a replacement for glial cells, which comprise the majority of brain cells.

Materials and methods

Cells and cell culture. The human glioblastoma cell line, U-251MG, was obtained from the JCRB Cell Bank (cat. no. IFO50288). The U-251MG cells are glial fibrillary acidic protein-positive glioblastoma multiforme derived from a grade III-IV astrocytoma in the brain of a 75-year-old patient, which was established as a cell line by Bigner *et al* (9) in 1981. The U-251MG cells express an α7 nicotinic acetylcholine receptor (10). The U-251MG cells were cultured in Eagle's

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minimum essential medium with L-glutamine (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% fetal bovine serum (Equitech-Bio. Inc.) and penicillin-streptomycin-amphotericin B suspension (FUJIFILM Wako Pure Chemical Corporation) at 37˚C with 5% CO_2.

Cell proliferation assay. The U-251MG cells were seeded at 2,000 cells/well in a 96-well culture plate (Sumitomo Bakelite Co., Ltd.) as independent wells with n=5 and pre-incubated for 24 h at 37˚C with 5% CO_2. A solution of (-)-nicotine (FUJIFILM Wako Pure Chemical Corporation) or (-)-cotinine (FUJIFILM Wako Pure Chemical Corporation) was applied to each well at 0-6 mM and incubated for 4 days at 37˚C with 5% CO_2. During the 4 days of incubation, the U-251MG cells were in the logarithmic growth phase. The medium was then replaced with 5% 2-(2-methoxy-4-nitroph enyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium solution (WST-8; Dojindo Laboratories, Inc.) diluted with an Eagle's minimum essential medium and incubated for 1.5 h at 37˚C with 5% CO_2. The absorbance was then measured at λ=450 nm using a multi-spectrophotometer (Viento; Dainippon Sumitomo Pharma, Co. Ltd.). The amount of formed formazan is proportional to the number of viable cells, as intracellular mitochondrial dehydrogenase reduces WST-8 to yellowish-orange formazan (11). Acetylcholine chloride (Nacalai Tesque, Inc.) and dopamine hydrochloride (Nacalai Tesque, Inc.) were also examined for comparison with nicotine.

Under the administration of Li_2CO_3, cell proliferation was examined following exposure to nicotine and cotinine. The U-251MG cells were seeded at 2,000 cells/well in a 96-well culture plate and pre-incubated for 24 h at 37˚C. In the experiment described above, a solution of 3.4 mM nicotine or 6 mM cotinine, which was found to decrease cell viability to ~70-80%, was applied to each well in combination with 0-1.5 mM Li_2CO_3 (Nacalai Tesque, Inc.) and incubated at 37˚C with 5% CO_2. Following 4 days of incubation, cell proliferation was measured using WST-8 assay. A solution of 6 mM acetylcholine and 60 μM dopamine was applied for comparison with nicotine.

To clarify the mechanisms of action of nicotine as regards cell proliferation, mecamylamine, a non-competitive antagonist of nicotinic acetylcholine receptors, was used in the experiments. The U-251MG cells were seeded at 2,000 cells/well in a 96-well culture plate and pre-incubated for 24 h at 37˚C. A solution of 20 μM mecamylamine hydrochloride (Cayman Chemical Company) was first applied to each well and incubated at 37˚C with 5% CO_2. Following a 5-h incubation, the medium was aspirated. Subsequently, 3.6 mM nicotine were applied to each well in combination with 20 μM mecamylamine hydrochloride and incubated at 37˚C with 5% CO_2. After 4 days of incubation, cell proliferation was measured using WST-8 assay as described above.

Determination of mitotic catastrophe in cells. For the evaluation of mitotic catastrophe, multinucleated cells with two or more nuclei were counted using nuclear staining (12). The U-251MG cells were treated with 3.4 mM nicotine or 6 mM cotinine in combinaion with or without 1 mM Li_2CO_3. Following 4 days of incubation for 24 h at 37˚C, the U-251MG cells were fixed with 4% paraformaldehyde at pH7.4 (FUJIFILM Wako Pure Chemical Corporation), and their cell nuclei were stained with Hoechst 33342 (Dojindo Laboratories, Inc.). Mitotic catastrophe in the U-251MG cells was observed and counted using a fluorescent microscope CKX-53 (Olympus Corporation) at an excitation/emission of 330-385 nm/420 nm and a magnification of x200. The ratio of cells undergoing mitotic catastrophe (%) was calculated for quantitative analysis on five fluorescence micrographs of each treatment.

Measurement of intracellular reactive oxygen species generation. The Nitroblue-tetrazolium (NBT) reduction assay was used to determine the production of superoxide anion radicals ($O_2^{\cdot-}$) in cells (13,14). The U-251MG cells were seeded at 6,000 cells/well in a 96-well culture plate as independent wells with n=5 and pre-incubated for 24 h at 37˚C with 5% CO_2. A solution of 3.4 mM nicotine or 6 mM cotinine was applied to each well in combination with or without 1 mM Li_2CO_3 followed by incubation for 1 day at 37˚C with 5% CO_2. The medium was then replaced with 0.2% nitrotetrazolium blue chloride (NBT, MilliporeSigma) solution and incubated at 37˚C with 5% CO_2. The 0.2% NBT solution was prepared at use, dissolved with an Eagle's minimum essential medium, and filtered through a 0.22-μm filter. Following a 5-h incubation, the absorbance of the NBT-formazan was measured at λ=620 nm using the multi-spectrophotometer (Viento). The U-251MG cells were fixed with 4% paraformaldehyde at pH7.4 and observed under a phase contrast microscope (CKX-53; Olympus Corporation) at x400 magnification. The amount of formed NBT-formazan is proportional to the amount of superoxide anion radicals ($O_2^{\cdot-}$) in cells. Cell proliferation was examined using WST-8 assay (as described above) in the same treatment groups, which confirmed that there were no differences between the groups (data not shown).

Statistical analysis. The data for cell proliferation, mitotic catastrophe and intracellular reactive oxygen species are expressed as the mean ± SD, as independent wells with n=5. The data were analyzed using one-way ANOVA followed by Dunnett's test. By contrast, the data for mecamylamine and NBT assay were analyzed using a Student's t-test with KaleidaGraph 4.5J software (HULINKS Inc.). A value of $P<0.05$ was considered to indicate a statistically significant difference. In the present study, experiments were repeated at least two times.

Results

Proliferation of human glioblastoma cells. The proliferation of the human glioblastoma U-251MG cells was markedly decreased to <16.6% at concentrations >2.4 mM nicotine (Fig. 1). Moreover, following exposure to cotinine, cell proliferation gradually decreased in a concentration-dependent manner to 73.8% with 6 mM cotinine. There was almost no decrease in cell proliferation following exposure to 0-6 mM acetylcholine (a neurotransmitter that interacts with nicotinic acetylcholine receptors). Cell proliferation was maintained at 83.5%, even at acetylcholine concentrations ≥4.8 mM. By contrast, cell proliferation was significantly decreased to <6.3% with dopamine at concentrations >60 μM. The

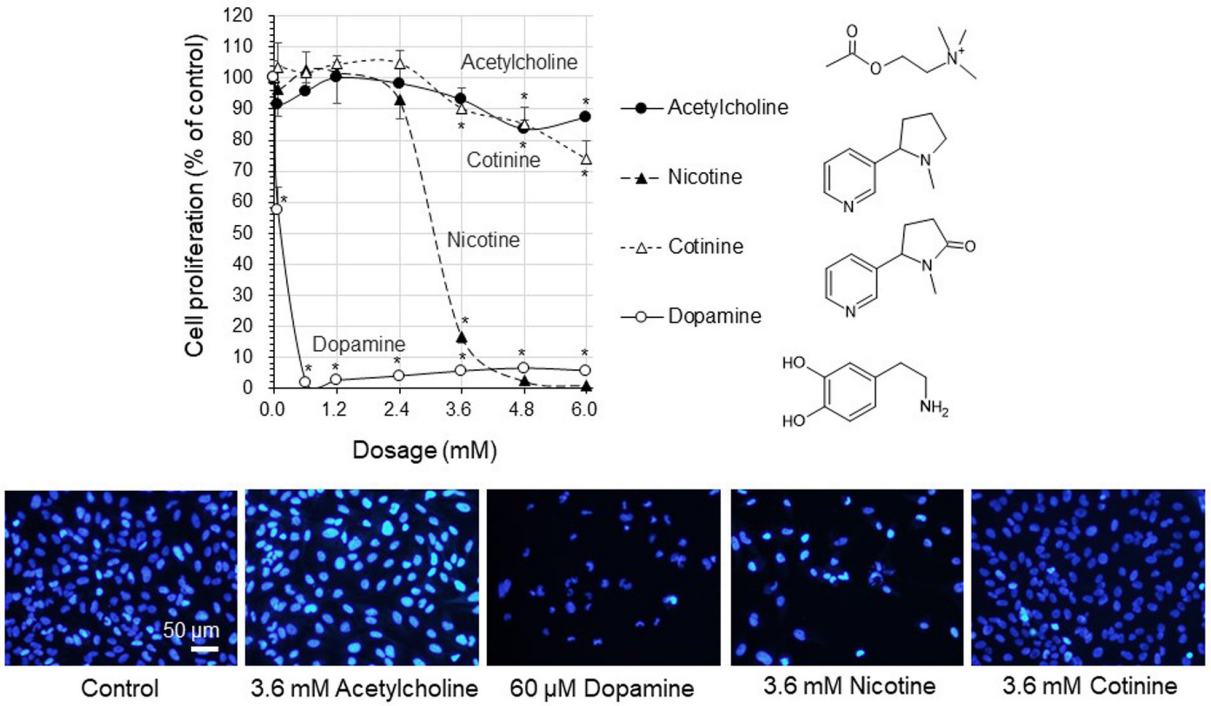

Figure 1. Proliferation of U-251MG human glioblastoma cells. The U-251MG cells were treated with 0-6 mM acetylcholine, dopamine, nicotine, or cotinine (chemical structures are presented in the top right panel). After 4 days of incubation, cell proliferation was measured using a mitochondrial dehydrogenase-reduced formazan-based WST-8 assay. The data are presented as the mean ± SD, n=5; *P<0.05 (vs. 0 mM). (Bottom panel) The cell nuclei were stained with Hoechst 33342, and cell morphology was observed using a fluorescence microscope at an excitation/emission of 330-385 nm/420 nm (magnification, x200). Scale bar, 50 μm.

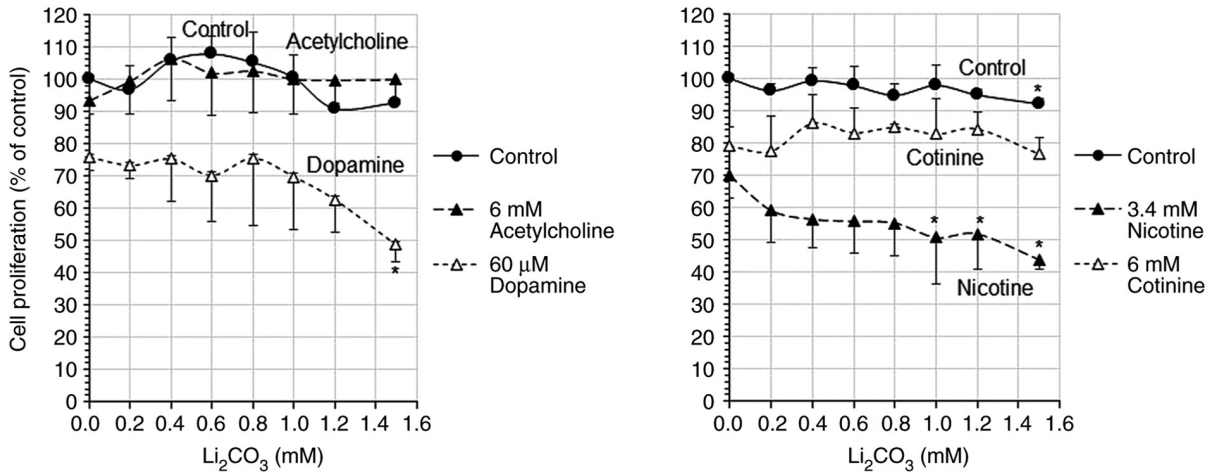

Figure 2. Proliferation of human glioblastoma U-251MG cells under lithium carbonate Li_2CO_3 administration. The U-251MG cells were treated with 6 mM acetylcholine, 60 μM dopamine, 3.4 mM nicotine, or 6 mM cotinine in combination with 0-1.5 mM Li_2CO_3. After 4 days of incubation, cell proliferation was measured using WST-8 assay. The data are presented as the mean ± SD, n=5; *P<0.05 (vs. 0 mM). Li_2CO_3, lithium carbonate.

microscopic observation clearly indicated that cell proliferation was not inhibited by acetylcholine and cotinine, whereas this decreased by dopamine and nicotine.

Following exposure to 0-1.5 mM Li_2CO_3, cell proliferation decreased from 75.8 to 48.6% in the cells treated with 60 μM dopamine, and from 69.8 to 43.9% in the cells treated with 3.4 mM nicotine in a concentration-dependent manner (with the increasing Li_2CO_3 concentration) (Fig. 2). By contrast, the cells treated with 6 mM acetylcholine or 6 mM cotinine exhibited a proliferation of 93.4-106.2 and 76.6-86.0%, respectively, which did not decrease even following exposure to high concentrations of Li_2CO_3. Exposure to 20 μM mecamylamine, a non-competitive antagonist of nicotinic acetylcholine receptors, restored the proliferation of the cells treated with 3.6 mM nicotine from 61.7 to 90.5% (Fig. 3).

Mitotic catastrophe in cells. The ratio of cells undergoing mitotic catastrophe was 3.5% in the controls; however, this increased to 5.2% in the cells exposed to 1 mM Li_2CO_3 (Fig. 4). In the cells treated with 3.4 mM nicotine, the ratio of cells

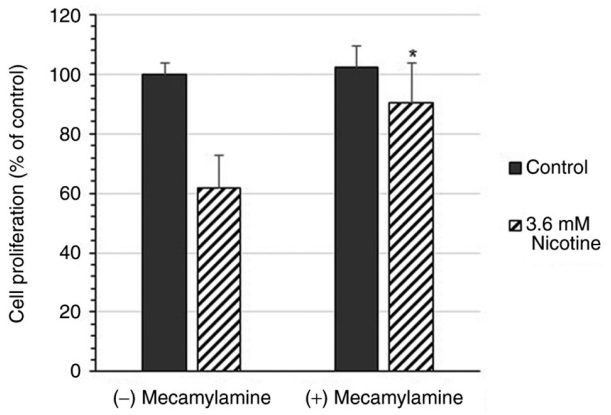

Figure 3. Restoration of cell proliferation by mecamylamine, a non-competitive antagonist of nicotinic acetylcholine receptors. The U-251MG cells were treated with 20 μM mecamylamine and 3.6 mM nicotine. After 4 days of incubation, cell proliferation was measured using WST-8 assay. The data are presented as the mean ± SD, n=5; *P<0.05 (vs. without mecamylamine).

undergoing mitotic catastrophe was significantly increased to 10.4, and to 14.4% in the cells treated with 3.4 mM nicotine in combination with 1 mM Li_2CO_3. Exposure to cotinine at 6 mM slightly increased the ratio of cells undergoing mitotic catastrophe to 4.9%, and the ratio remained relative unaltered following the administration of 1 mM Li_2CO_3.

Intracellular reactive oxygen species generation. The amount of $O_2^{\cdot -}$ (% of control) was slightly decreased to 93.4% in the cells treated with 1 mM Li_2CO_3 (Fig. 4). In the cells treated with 3.4 mM nicotine, the amount of $O_2^{\cdot -}$ was 109.4%, and this was increased to 110.4% in the cells treated with 3.4 mM nicotine in combination with 1 mM Li_2CO_3. Moreover, the cells treated with 6 mM cotinine exhibited almost no increase in the amount of $O_2^{\cdot -}$ at 102.8%, which was almost the same as the control even under the administration of 1 mM Li_2CO_3.

Discussion

The present study examined the effects of nicotine and its metabolite, cotinine, on the proliferation of U-251MG cells exposed to Li_2CO_3, and compared these effects to those of acetylcholine and dopamine. The results indicated that the proliferation of U-251MG human glioblastoma cells was markedly decreased at high concentrations of nicotine. Moreover, cell proliferation was gradually decreased in a concentration-dependent manner with cotinine. Previous studies have demonstrated the actual blood nicotine and cotinine levels of smokers. Blood nicotine concentrations have been shown to vary from 25 to 444 nmol/l, and the average was found to be 203 nmol/l in 206 females and 126 males (15). In human serum samples from smokers, the concentrations of nicotine and cotinine have been found to be 49-92 and 885-2111 nmol/l, respectively (16). Of note, the concentrations of nicotine and cotinine used in the present study were significantly higher than the blood levels of actual smokers, which allowed for the clarification of the differences in the biological effects of nicotine and cotinine. Cotinine is the major metabolite of nicotine (17), with a half-life of 10 to 30 h, while nicotine has a half-life of 0.5 to 2 h (18). Riah *et al* (19) reported that nicotine was 100-fold more toxic than cotinine, and 10-fold more rapid than cotinine at producing respiratory arrest in mice. Similarly, the results of the present study demonstrated that the cytotoxicity of nicotine outweighed that of cotinine. There was almost no decrease in cell proliferation following the exposure of the cells to acetylcholine (a neurotransmitter that interacts with nicotinic acetylcholine receptors). The microscopic examination clearly revealed that acetylcholine and cotinine did not inhibit cell proliferation, whereas dopamine and nicotine decreased cell proliferation. It was also observed that cells treated with dopamine or nicotine underwent mitotic catastrophe. In the present study, following exposure to 0-1.5 mM Li_2CO_3, cell proliferation decreased in the dopamine- and nicotine-treated cells in a concentration-dependent manner (with the increasing concentrations of Li_2CO_3). By contrast, the cells treated with acetylcholine or cotinine did not exhibit a decrease in proliferation, even at high concentrations of Li_2CO_3. de Sousa *et al* (20) demonstrated that lithium treatment increased mitochondrial electron transport complex I activity in leukocytes of subjects with bipolar disorder. The lithium carbonate, Li_2CO_3, has been used as a mood stabilizer in bipolar disorder at a dose range of 0.6-1.0 mM and >1.5 mM as lithium in serum, which is close to the addiction level of Li_2CO_3 (7). In the present study, following exposure to 0-1.5 mM Li_2CO_3, cell proliferation decreased in the dopamine- and nicotine-treated cells in a concentration-dependent manner (with the increasing concentrations of Li_2CO_3). By contrast, cells treated with acetylcholine or cotinine did not exhibit a decrease in growth, even with high concentrations of Li_2CO_3. These results suggest that under Li_2CO_3 administration, the increase in nicotine and dopamine levels due to smoking may increase health risks through cytotoxicity.

It is known that nicotinic acetylcholine receptors are activated by acetylcholine or exogenous ligands, such as nicotine (21). Nicotine administration increases the firing ratio of substantia nigra pars compacta neurons and induces striatal dopamine release (2,3). It has been found that dopamine is an essential endogenous regulator of malignant glioma growth (22), and dopamine increases apoptosis by inducing mitochondrial dysfunction (22). The findings of the present study are consistent with these findings in that dopamine induced a decrease in cell proliferation.

The physiological effects of nicotine are mediated mainly by nicotinic acetylcholine receptors, expressed in human brain cells and on the surface of the pancreas, colon, bladder, airway epithelia, etc. (23-25). The U-251MG cells express an α7 nicotinic acetylcholine receptor (10). The role of nicotinic acetylcholine receptors can be assessed by the co-incubation of nicotine and a non-competitive nicotinic acetylcholine receptor antagonist (26). In the present study, mecamylamine, a non-competitive antagonist of nicotinic acetylcholine receptors, restored cell proliferation which had been reduced by nicotine, suggesting that a decrease in cell proliferation was induced by nicotine via nicotinic acetylcholine receptors.

The ratio of cells undergoing mitotic catastrophe was significantly increased in the cells treated with nicotine in combination with Li_2CO_3. Cotinine slightly increased the ration of cells undergoing mitotic catastrophe, and the ratio was almost the same under the administration of Li_2CO_3. It has been reported that 10 μM nicotine-induced mitochondrial

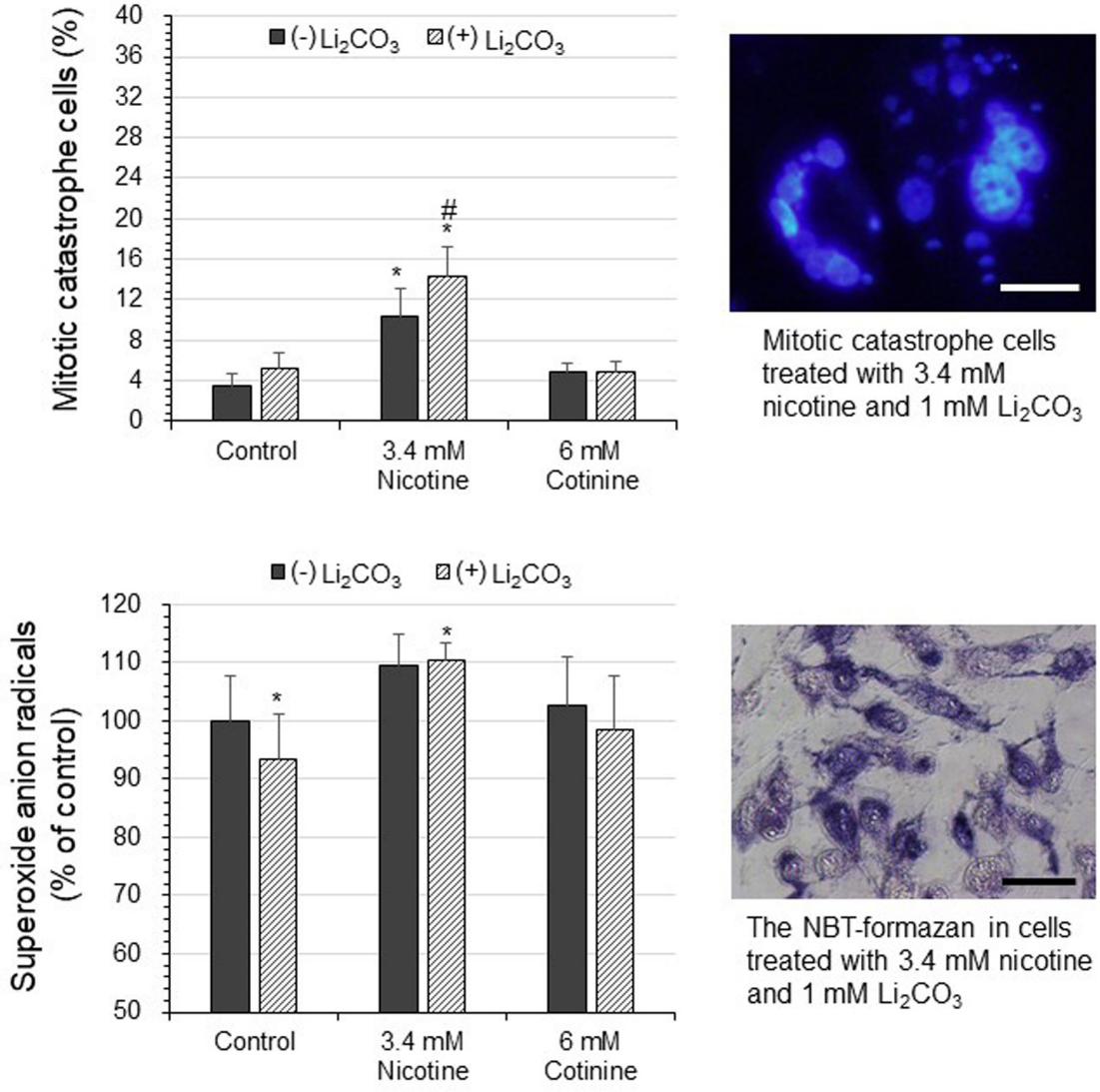

Figure 4. Mitotic catastrophe and intracellular reactive oxygen species in human glioblastoma U-251MG cells under Li_2CO_3 administration. The U-251MG cells were treated with 3.4 mM nicotine or 6 mM cotinine in combination with or without 1 mM Li_2CO_3. After 4 days of incubation, the cell nuclei were stained with Hoechst 33342. The mitotic catastrophe cells (%) ratio was calculated on five fluoresce micrographs of each treatment. The data are presented as the mean ± SD, n=5; $^*P<0.05$ (vs. control without Li_2CO_3), $^\#P<0.05$ (vs. without Li_2CO_3). The absorbance was measured at an excitation/emission of 330-385 nm/420 nm (magnification, x200). Scale bar, 20 μm. In the other experiments, the U-251MG cells were treated as mentioned above, and after 1 day of incubation, superoxide anion radicals were measured using NBT assay. The data are presented as the mean ± SD, n=5; $^*P<0.05$ (vs. control without Li_2CO_3). Magnification, x400; scale bar, 40 μm. Li_2CO_3, lithium carbonate; NBT, nitroblue-tetrazolium.

fragmentation and cell growth inhibition in a nicotinic acetylcholine receptor-dependent manner in NT2/D1 human multipotent embryonic carcinoma cells (27). Pastor *et al* (28) indicated that Li_2CO_3 exerted a cytotoxic effect in Chinese hamster repair-deficient mutant AA8 CHO cells, mitotic abnormalities such as multipolar anaphases and lagging chromosomes, leading to micronuclei. Cell proliferation represents an increase in cell number resulting from regulated cell growth and cell division. The results of the present study suggested that nicotine caused mitotic catastrophe, which was amplified under Li_2CO_3 administration, and the suppression of cell proliferation was attenuated when nicotine was metabolized to cotinine.

As regards the biological effects of nicotine, there are reports that nicotine increases oxidative stress, or conversely, nicotine reduces oxidative stress. In the mitochondrial and microsomal compartments of the rat brain, nicotine-induced oxidative stress is accompanied by increasing levels of thiobarbituric acid-reactive substances and 4-hydroxynonenal specific glutathione-*S*-transferase activity (29). However, Cormier *et al* reported that nicotine bound to complex I of the mitochondrial respiratory chain and inhibited NDH-ubiquinone reductase activity, resulting in decreased levels of $O_2^{\cdot-}$ in rat brain mitochondria (30). In the present study, the amount of $O_2^{\cdot-}$ (% of control) was slightly decreased in the cells treated with Li_2CO_3. In the cells treated with nicotine in combination with Li_2CO_3, the amount of $O_2^{\cdot-}$ was increased by 10%. Moreover, cotinine led to almost no increase in the amount of $O_2^{\cdot-}$, the levels of which were almost the same as those of the control, even under the administration of Li_2CO_3. These results suggest that nicotine increases the amount of $O_2^{\cdot-}$ regardless of Li_2CO_3 administration, and that when nicotine is metabolized into

cotinine, the increase in $O_2^{\cdot-}$ generation is attenuated. The formation of $O_2^{\cdot-}$ was slightly reduced with 1 mM Li_2CO_3 in the controls, probably due to the reducing ability of lithium. The suppression of cell proliferation by nicotine may involve both nicotine-induced cytotoxicity and the increase in reactive oxygen species, which is not canceled by Li_2CO_3.

Overall, the results of the present study suggested that nicotine in combination with Li_2CO_3 suppressed the proliferation of human glioblastoma cells, accompanied by mitotic catastrophe and $O_2^{\cdot-}$ generation. Increased mitotic catastrophe and $O_2^{\cdot-}$ production suggest that cellular dysfunction has occurred. The cytotoxicity of cotinine is lower than that of nicotine. However, nicotine is metabolized into cotinine by the hepatic cytochrome, P450 2A6 enzyme (31). Smoking is one of the factors that impair hepatic function (32). Under lithium carbonate administration, it is advisable to refrain from smoking as much as possible to avoid the health risks associated with nicotine.

In conclusion, the present study demonstrated that nicotine significantly decreased cell proliferation via nicotinic acetylcholine receptors, and cell proliferation was further suppressed by the combined administration of Li_2CO_3 in U-251MG human glioblastoma cells. As opposed to nicotine, the suppression of cell proliferation was less observed in the cells treated with cotinine. As regards endogenous neurotransmitters involved in nicotinic acetylcholine receptors, acetylcholine exerted no cytotoxic effects, whereas dopamine suppressed cell proliferation under Li_2CO_3 administration. These findings provide cellular biological insight into the risks of smoking under Li_2CO_3 administration. However, since the present study was an *in vitro* study, no conclusions could be drawn about the association between psychiatric patients and smoking by extrapolating these data, and thus, further *in vivo* studies are warranted.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

SK was involved in the conceptualization, methodology, investigation and writing of the study. SK confirms the authenticity of all the raw data. The author has read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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