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Original Article

Assessment of Potential *In Vitro* Genotoxic and Cytotoxic Effects of Bupropion Hydrochloride (Wellbutrin) in Human Peripheral Lymphocytes and Human Cortical Neuron

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ABSTRACT

Introduction: Wellbutrin (bupropion hydrochloride; WB), an anti-depressant of the aminoketone class is new highly selective norepinephrine and dopamine reuptake inhibitor; it is effective in the treatment of patients with major depression. **Materials and Methods:** To investigate the *in vitro* effects of WB in human cultured peripheral blood lymphocytes and human cortical neural (HCN2) cell lines, micronucleus, sister chromatid exchange analysis, cellular viability, and comet assays were employed. The present study is to our knowledge, the first report on WB genotoxicity in cultured human peripheral blood lymphocytes and its cytotoxicity in the HCN2 cell line. We have also investigated the genotoxic potential of WB to induce chromosomal aberrations. **Results:** WB-induced cytotoxicity (measured as reduction of the nuclear division index) possibly prevented the division of damaged cells. **Conclusion:** We conclude that although, WB exerts potential genotoxic effects in cultured lymphocytes, its cytogenetic effects are very unlikely to occur in blood cultures of WB-administered subjects.

Key words: Comet assay, genotoxicity, micronucleus, wellbutrin

INTRODUCTION

Depression is a state of mental illness and has been portrayed in different ways; in the 20th century, it was categorized into endogenous and neurotic forms. Some depressive disorders such as bipolar disorder, although, less common are shown to be inherited.[1]

Anti-depressant drugs reduce and sometimes even eliminate symptoms of depression. A variety of such drugs are available, which include the monoamine oxidase inhibitors, tricyclic anti-depressants and in more recent times, selective serotonin reuptake inhibitors and selective norepinephrine reuptake inhibitors.[1]

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Bupropion hydrochloride (commercially, Wellbutrin [WB]) is an antidepressant with a dual effect on norepinephrine and dopamine neurotransmitter systems. In 1996, a sustained release product was introduced and an extended release formulation of Bupropion was approved for use in 2003. Metabolites of bupropion, hydroxybupropion, and theohydrobupropion have been reported to lower the uptake of dopamine and norepinephrine in mouse synaptosomes.[2] An overdose of bupropion results in significant clinical effects in over 1/3rd cases; affected individuals commonly manifest symptoms such as sinus tachycardia, hypertension, drowsiness, lethargy, agitation, nausea, and vomiting. It has also shown to cause mutagenic effects, cytotoxicity and chromosomal abnormalities, including, gaps, deletions, breaks and

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polyploidy in mouse sperms and bone marrow cells.[3] In the present study, we have observed that WB is capable of producing deoxyribonucleic acid (DNA) damage in human lymphocytes. The genotoxicity of WB was cytogenetically tested by assessing its ability to induce sister chromatid exchange (SCE), DNA strand breakage and Micronucleus (MN) in cultured peripheral lymphocytes of normal individuals. The frequency of SCE did not show a significant difference. However, a statistically significant increase in the frequency of MN was detected in treated individuals. Previous studies have shown non-random distributions in the observed number of structural chromosomal aberrations (CAs).[4,5] Such results indicate that non-random processes may underlie the formation of CA (breaks and gaps) and give rise to MN.[6-11] In this study, we assessed whether a non-random process may contribute to WB-induced MN formation *in vitro*.

Genotoxicity testing of pharmaceuticals prior to commercialization is mandated by regulatory agencies world-wide. From a drug development standpoint, it is important to thoroughly understand the mechanisms of any positive genotoxic effects, and hence that informed decisions may be made with respect to risks.[12,13]

MATERIALS AND METHODS

Test drug and chemicals

WB (bupropion hydrochloride, $C_{13}H_{18}ClNOHCl$, Glaxo Smith Kline, USA is an antidepressant of the aminoketone class, and is chemically unrelated to other known antidepressants. The structure of WB closely resembles that of diethylpropion and is related to phenylethylamine, which is designated as (±)-1-(3-chlorophenyl) 1-2-([1,1-dimethyl ethyl] amino)-1-propanone hydrochloride [Figure 1a]. It was dissolved in 8% dimethylsulfoxide, obtained from Merck, USA. Cytochalasin-B (Cyt-B) and colcemid were purchased from Sigma-Aldrich, India. Other chemicals for fixation and staining were obtained from Merck, USA.

Donors and collection of blood samples

The study was carried out by using blood samples from four healthy, non-smoking donors (two males and two females), aged between 20 years and 35 years. All volunteers gave informed consent to participate in the study. All the test conditions to use human samples have been approved by University ethical committee. The criteria of acceptability to ensure reliability of the experiment were: Having good health, not having serious illness and receiving any medical therapy, not taking alcohol or drugs, or smoking cigarettes. All blood samples were taken between 9:00 am and 9:30 am on the same day of initiation of the experiment, to minimize any confounding effects of dietary factors.

Lymphocyte cultures

Venous blood was drawn in sterile condition from each subject and whole culture were set up in the triplicates by adding 0.6 ml of heparinized blood to 6 ml of Roswell Park Memorial Institute (RPMI 1640) medium (Gibco, USA), supplemented with 1.2 ml of fetal bovine serum (Hi-media, India), antibiotics (penicillin and streptomycin) (Hi-media, India) and the lymphocytes were stimulated with 4% phytohemagglutinin (PHA; Invitrogen, USA).

For chromosome aberration analysis, the cultures were incubated at 37°C for 72 h. One hour prior to harvest, 0.4 μg/ml of colchicine (Sigma-Aldrich, USA) was added to arrest the cells at metaphase. Culture was incubated at 37°C for 72 h prior to cell harvest. During harvesting time culture was not allowed to expose to any source of light. The harvesting procedure remains same as chromosome aberration analysis and slides were prepared by dropping the cells in wet slides. For the MN assay, the cultures were also incubated at 37°C for 72 h. BN cells were accumulated by adding Cyt-B (Sigma-Aldrich, USA) at a final concentration of 6 μg/ml at 48 h following initiation of culture. At the end of the incubation time, the cells were collected after centrifugation at 1000 rpm for 5 min, for chromosome aberration analysis, cells were re-suspended in a hypotonic solution (0.075 M KCl, Himedia, India) for 6 min and fixed three times in a Carnoy's fixative. Cells were spread on slides and stained with Giemsa (Himedia, India). For the MN assay, cells were washed once in RPMI 1640 medium and then a mild hypotonic treatment was given for 2-3 min at room temperature. The cells were then centrifuged in 1000 rpm for 3 min and fixed with a Carnoy's fixative. The fixation step was repeated twice. Air-dried preparation was made and stained with Giemsa.

In this study, test concentrations of WB were chosen on the basis of a previous cytotoxicity test based on mitotic index (MI), which provides reliable information about the effects of solvent and test substance on metaphase chromosomes. According to the cytotoxicity test procedure, we prepared a series of blood lymphocyte cultures of WB. Cultures were treated with WB for both 24 h and 48 h. Besides, if the chemical is unstable, the cells are exposed to it only until the G_0/G_1 phase, which for some chemicals, may be a relatively insensitive stage of the cell cycle.[14] Lymphocyte cultures have over half their metaphases in the second division at 48 h.[15] Therefore, the best period to culture human peripheral lymphocytes for chromosome analysis in subjects exposed to mutagens and carcinogens is 44-48 h.

Alkaline single-cell gel electrophoresis (comet assay)

Comet assay detects DNA damage through electrophoresis[14] and subsequent staining in N', N'-dimethyl-N-[(E)-(3-methyl-1, 3-benzothiozol-2-ylidene)

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methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1, 3-diamine (commercially named as SYBR green) dye. Treated cells were harvested and washed twice in phosphate buffer saline (PBS) and processed according to the protocol given by Poddar *et al*.[14] The slides were air-dried and stained with propidium iodide (1:1000) and analyzed under fluorescent microscope. The tail moments of the nuclei were measured as a function of DNA damage. Analysis was carried out using comet imager v1.2 software (Metasystems, Germany); 50 comets were analyzed per concentration.

Cell culture and cellular viability determination

The cell line human cortical neural (HCN2) (American Type Culture Collection, VA) were thawed at 37°C and re-suspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (pH 7.35) and the cells were maintained at 37°C in 5% CO_2. The neurons were differentiated in this media for 3 h, by addition of 5 mM retinoic acid (Sigma-Aldrich, USA) for 4-5 days (this results in morphologic changes, which include the development of neuritic processes, as well as the expression of neuronal markers) by which time, they are known to develop long branched neuritic processes, resemble mature neurons and do not change their morphology over time. Once the neurons were differentiated and developed neuritic processes, cells were treated with 100 μM WB for 24 and 48 h. The cells were fixed with ice-cold 90% (v/v) methanol (−20°C) for 20 min and stained with coomassie brilliant blue. Control and WB-treated cells were washed twice with PBS and incubated for 3 h with 55 μl of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution (5 mg/ml). Formation of formazan crystals was detected at 570 nm and cellular viability was expressed as % reduced MTT in comparison with control values. MTT activity was expressed as percentage of control and data was analyzed using one-way ANOVA with $P \leq 0.05$.

Sister chromatid exchange analysis

For SCE analysis, Bromodeoxy uridine (BrdU; Sigma-Aldrich, India) was added after 24 h at a final concentration of 20 μM. At the start of the culture, blood was treated with WB; the culture was incubated at 37°C for 72 h prior to cell harvest. SCEs were visualized after staining by a modified method of Perry and Wolff.[16] Toxicity was determined by scoring first (M1), second (M2) and third division mitoses (M3) among 100 metaphases and calculating the proliferation index (PI) using the formula: PI = (M1 + [2M2] + [3M3])/100.

Cytokinesis-block micronucleus assay

The CBMN assay was carried out using techniques of Fenech.[17] Cultures were incubated at 37°C for 68 h. WB was added at 20 and 44 h after initiation of the cultures. Cyt-B (final concentration of 8 μg/ml) was added at 44 h when the cultures were established, to arrest cytokinesis and obtain binucleated (BN) cells. The cells were harvested and processed as described earlier.[18]

Slide scoring and statistical analysis

For SCE analysis, a minimum of 50 well spread metaphases were recorded for each of the patients' samples. The criteria suggested by Obe *et al*.[19] were applied for scoring. Every switch of staining between the sister chromatids was scored as an SCE. Results were found to be insignificant by Fisher's exact test when compared to the controls.

For the MN assay, a total of 2000 BN cells with well-defined cytoplasm were examined for each individual on coded slides. The MN was identified according to the criteria described by Fenech.[17] In addition, a minimum of 500 cells was also scored to determine the percentage of cells with 1, 2, 3 and >4 nuclei. A nuclear division index (NDI) was calculated as per the formula NDI = (M1 + 2M2 + 3M3 + 4M4)/n; M1 to M4 indicates the number of cells with one to four nuclei, respectively, and n is the number of cells scored.

RESULTS

Deoxyribonucleic acid damage analysis

Figure 1b summarizes the induction of WB mediated effects in human blood samples (mean of five experiments with blood from different donors). WB-induced a concentration-related reduction resulting DNA migration. A significant difference was obtained with WB concentrations of 25 μM and higher. DNA damage induced in blood samples at the start of the culture are removed in time [Figure 1c]; 8 h after initiation of the culture, DNA damage induced by 100 μM WB is completely removed, whereas, portions of DNA damage induced by higher WB concentrations (200 and 300 μM) still persist after 24 h as shown in Figure 1c and d.

Sister chromatid exchange analysis

SCEs were significantly induced by WB in human blood cultures [Figures 2a and b] treated at the start of the culture at a WB concentration of 200 μM [Figure 2c]. However, this treatment strongly reduced the PI of the blood cultures, indicating a clear cytotoxic effect [Figure 2d]. Figure 2d also depicts that cytotoxicity (reduction in the PI) is already seen at 100 μM WB. Thus, it can be concluded from these experiments that WB-induced cytotoxic effects occur in parallel or even precede genotoxic effect in the SCE test under these experimental conditions.

Micronucleus test

Figure 3a-i summarizes results of the MN assay. WB

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treatment of blood samples at the start of the culture did not lead to increased MN frequencies in BN cells [Figures 3a and d]. WB concentrations up to 250 μM (which already caused strong cytotoxic effects measured as a reduction in the NDI, Figure 3g) could be tested. Higher concentrations totally inhibited proliferation of lymphocytes. When blood cultures were treated with WB 24 h after the start of the cultures, WB concentrations up to 400 μM could be tested; however, no significant induction of MN was measured [Figures 3b and e]. In the experiments with 400 μM, <1000 cells have been scored in the four cultures of two experiments. The figure is based on a total of 3825 cells. Figure 3h clearly indicates that the NDI greatly decreases under these experimental conditions. The use of 500 μM WB led to a complete failure of two out of the three tests. The results of the experiments suggest that WB induces a strong cytotoxic effect under these experimental conditions, however, causes no significant induction of MN. When blood cultures were treated with WB at 44 h after the start of the cultures, Clear, concentration-related induction of MN is observed [Figure 3c and f]. A statistically significant induction of MN is measured at concentrations of 300 μM and higher. In the experiments with 400 μM WB < 1000 BN cells (between 562 and 938) were scored in five out of the six cultures (a total of 4322 cells). Decrease in the NDI as an indicator of cytotoxicity parallels the induction of MN [Figure 3i].

Cytotoxicity assays

The human cortical neuronal cells were treated with WB. In order to determine the time at which this drug can cause loss of neuritis in human neurons, morphology studies were done. The untreated (control), differentiated HCN2 cells developed a neuronal phenotype by extending long neuritic processes [Figure 4a and b]. When HCN2 cells were treated with 100 μM WB for 48 h, a large number of cells were found to be floating in the wells indicating cell death at this

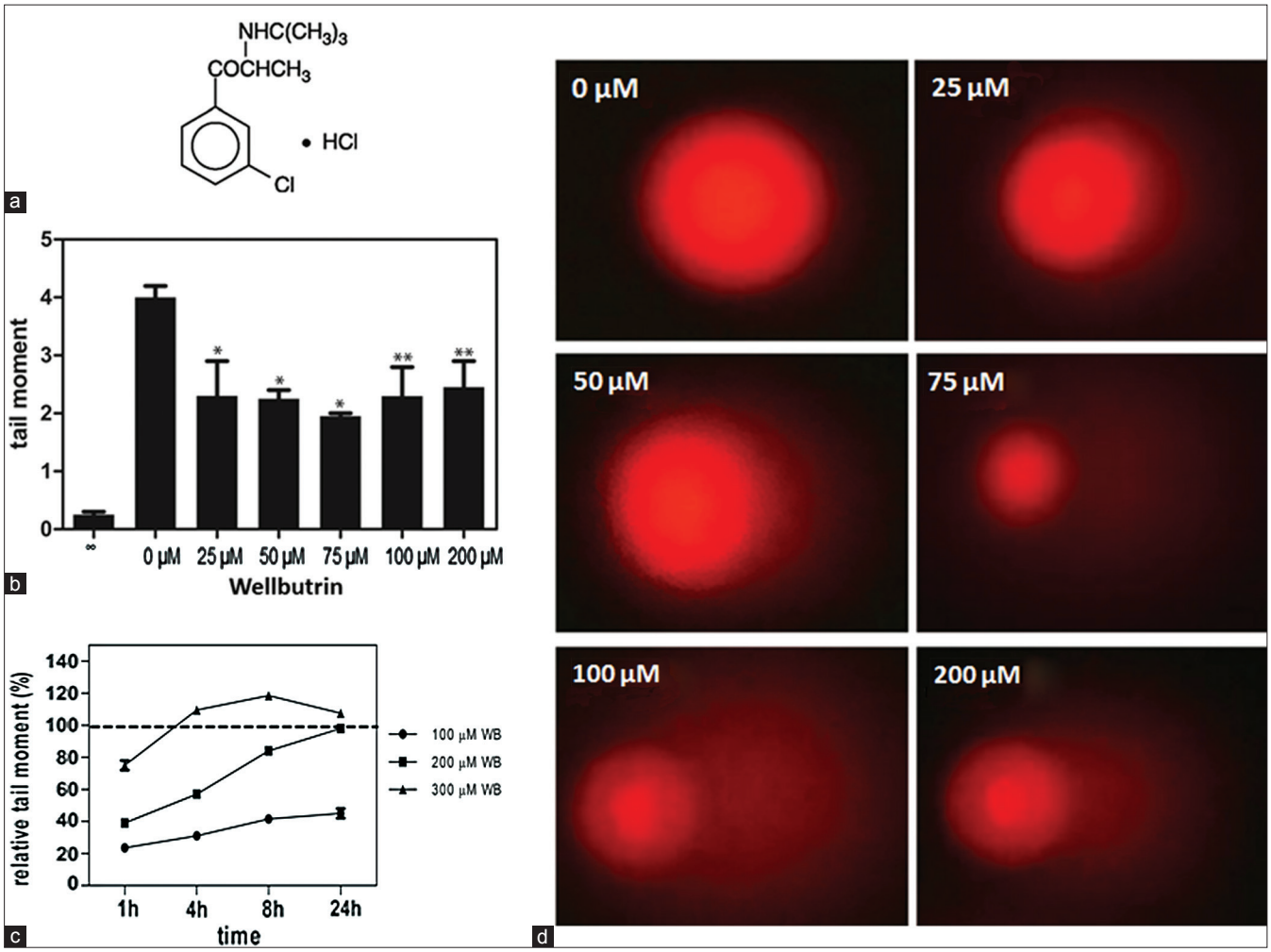


Figure 1: (a) Chemical structure of wellbutrin (WB) (b) Induction of deoxyribonucleic acid (DNA) damage by WB in human whole blood cultures. WB-induced DNA migration (tail moment [TM]) by increasing WB concentrations. Mean ± SEM of three independent tests with blood from different donors. **P*<0.05; ***P*<0.01 (c) Removal of WB-induced DNA damage in human blood cultures in time. Cancellation of the reduction of WB-induced DNA migration (relative TM; effect of WB alone corresponds to 100%). Mean of three independent tests with blood from different donors (d) Cells treated with increasing concentration of WB (stained by proliferation index)

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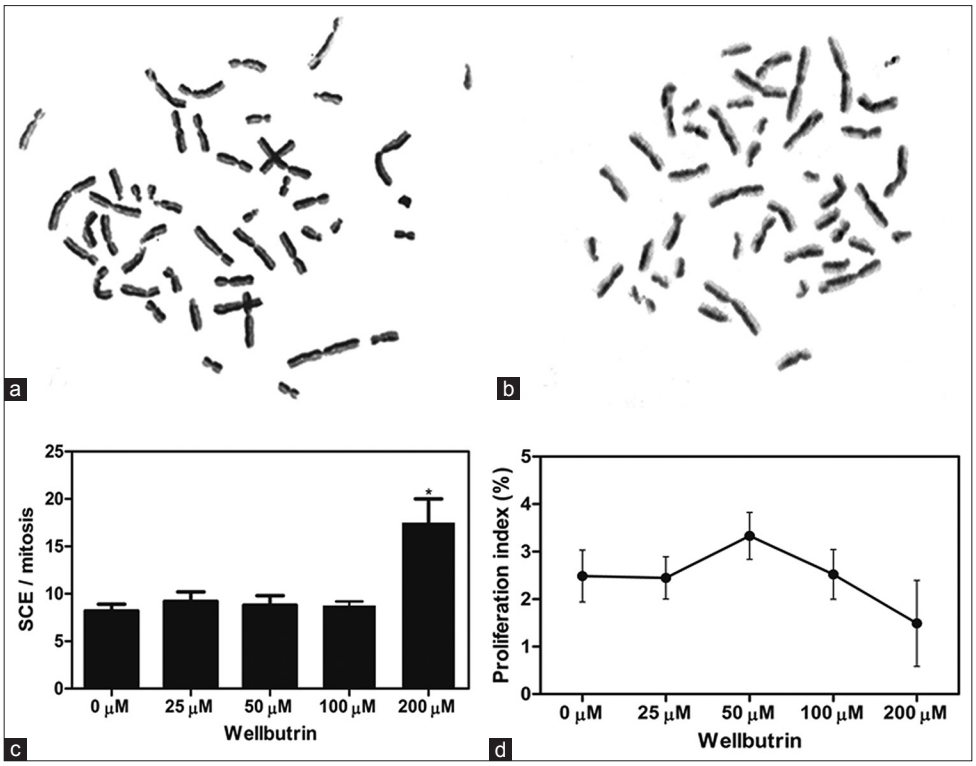


Figure 2: Induction of sister chromatid exchange (a) Metaphase showing SCE treated by 0 μM wellbutrin (WB) and (b) by 50 μM WB (c) in human whole blood cultures treated with WB at the start of the cultures. (d) The proliferation index (Percent change following the exposure to WB). Mean±SEM of three independent tests with blood from different donors. *$P < 0.01$

time. The cells that had survived after 48 h of treatment exhibited substantial neurite loss [Figure 4c and d].

In order to confirm the impact of WB on cell death in HCN2 cells, cellular viability studies were performed using the MTT assay [Figure 4]. The MTT assay was used to quantify the relative viability of HCN2 after WB treatment. This colorimetric assay corroborated our morphological study. When the cells were treated with 100 μM WB for 24 h, significant cell death was not observed. However, significant cell death was noted in HCN2 cells that were treated with WB for 48 h. These results further explained the large numbers of floating or dead cells that were noted in the morphology studies at this time point.

DISCUSSION

Our results clearly indicate that WB induces DNA damage in human blood cells and that WB-induced DNA damage can lead to the formation of SCE and MN under certain conditions. DNA damages were detected by the comet assay after treatment with 25 μM WB. However, several studies have suggested clastogenic effect of certain drugs in human peripheral lymphocytes.[20-23] A similar sensitivity of the comet assay for the detection of WB-induced DNA damage in other drugs has been reported for other mammalian cells.[24] Although, conflicting results exist and a critical assessment of these studies (positives and negatives) is hampered, partly by the incomplete information on exposure, and owing to lack of details of the study design and the methodology, the positive findings pose a matter of concern for the evaluation of the genotoxicity of WB.

After exposure of human subjects to WB *in vivo*, SCEs and MN in BN cells (BNMN method) only develop during the culture period of the blood samples, as a consequence of WB-induced DNA damage. We therefore, tried an *ex vivo* approach to assess the extent of DNA damage seen at the start of the culture (i. e., the time of blood sampling in biomonitoring studies) that is necessary to induce cytogenetic effects in cultured blood samples. In accordance with previously published data,[18] we may affirm that DNA damages are removed from blood cells during the culture period. SCEs are induced when DNA damages persist until S-phase of stimulated lymphocytes. At a concentration of 200 μM, a significant amount of DNA damage persisted and SCEs were induced. Induction of SCE was accompanied by a strong cytotoxic effect, which actually seems to occur at lower concentrations than at concentrations where genotoxic effects are exerted by WB. We can assume that DNA damage is also a plausible cause for the cytotoxic effect; WB-induced cytotoxicity may also be based on additional mechanisms (e.g., reactions of the drug with cellular proteins). Taken together, these experiments suggest that the induction of SCEs by WB in human blood cultures requires high, cytotoxic WB concentrations (200 μM) that

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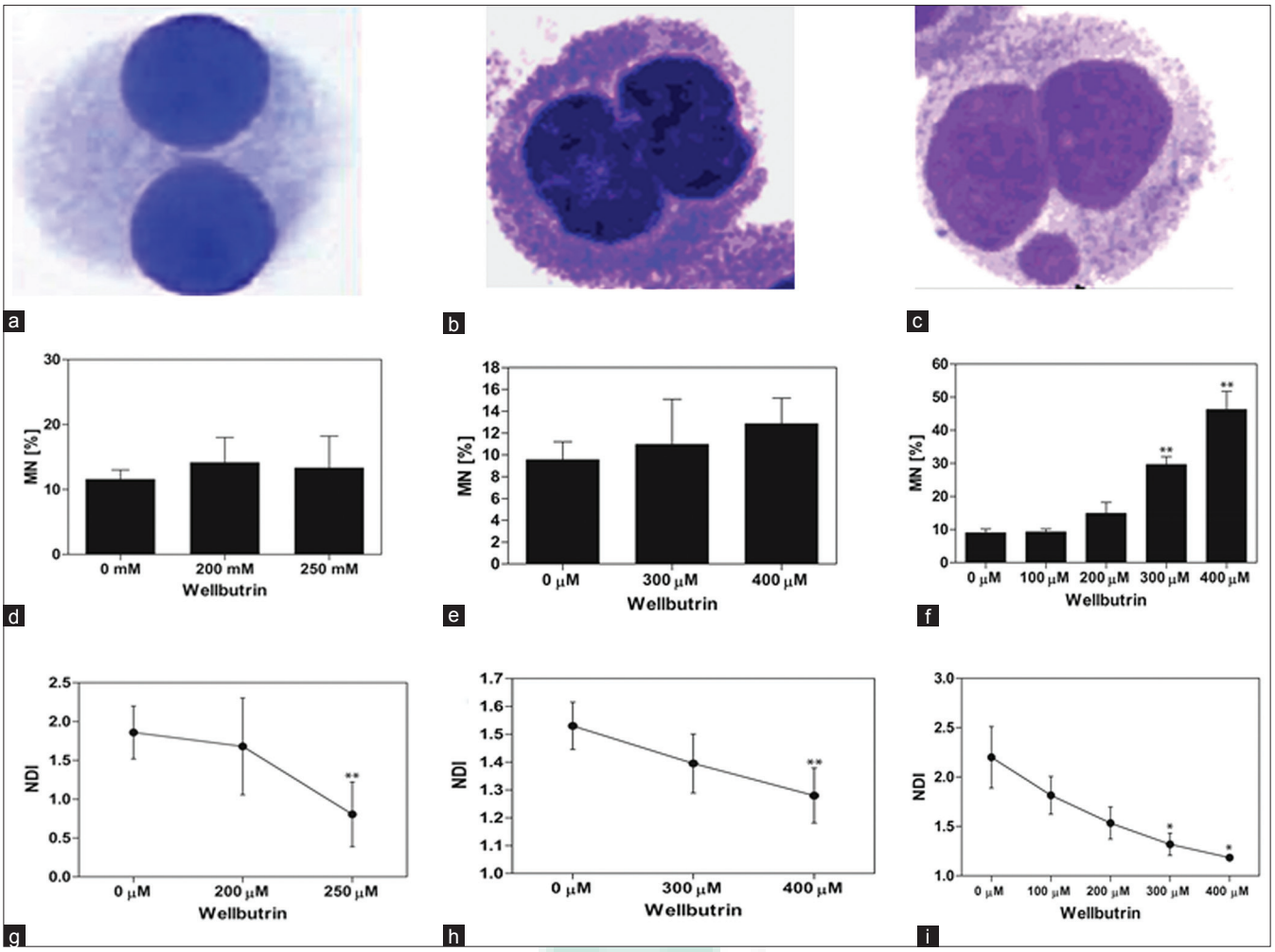


Figure 3: Cells treated with wellbutrin (WB) at (a) start of the cultures (b) after 24 h and (c) after 44 h (d) induction of micronucleus in three different concentrations at start of the cultures and (g) reduction of the nuclear division index (NDI), (e) after 24 h and (h) reduction of the NDI, (f) after 44 h and (i) reduction of the NDI. Mean±SEM of three independent tests with blood from different donors. **P*<0.05; ***P*<0.01

cause enough DNA damage that persists until replication phase. Such extents of DNA damage cannot be expected in blood cells of humans occupationally exposed to WB.[25]

WB did not induce MN in human blood cultures treated at the start of the culture. It is likely that low concentrations did not induce enough DNA damage to induce MN; high concentrations prevented damaged cells from progressing beyond mitosis under these experimental conditions. The criteria suggested by organization for economic corporation and development (OECD) for the *in vitro* MN test suggests adding the test substance 24 h after the start of the blood cultures (to add CytB 20 h later and to prepare the slides 72 h after the start of the cultures). Considering this approach, we were able to test higher WB concentrations (up to 400 µM), but did not measure a significant increase in the MN frequency. Again, considerable cytotoxicity was obvious under these test conditions. The OECD draft guideline for the *in vitro* micro nucleus test (MNT) recommends to test substances with an equivocal or negative response with a modified protocol and to add the test compound (together with the CytB) 48 h after the start of the blood culture. This protocol is expected to be most sensitive, since proliferating lymphocytes are treated during the last cell cycle before preparation. We could demonstrate that WB induces MN in human whole blood cultures when added at this late time point (i.e., treating the proliferating cells during their last cell cycle before preparation). Nevertheless, cytotoxic effects were obvious under these experimental conditions as well. On the whole, these experiments clearly indicate that WB is a weak inducer of MN in human blood cultures, and that the induction of MN in human blood cultures requires specific conditions.

Anti-depressant WB has been implicated in causing disruption of neurite morphology, ultimately leading to cell death. On the basis of cell survival studies and morphology studies, it was observed that at 24 h, there was some disruption in neurite morphology, while significant cell death was not observed until 32-36 h. Because the cytoskeleton plays a role in neurite outgrowth, the effect of oxidative stress on the cytoskeletal structures was observed at earlier (42 h) and

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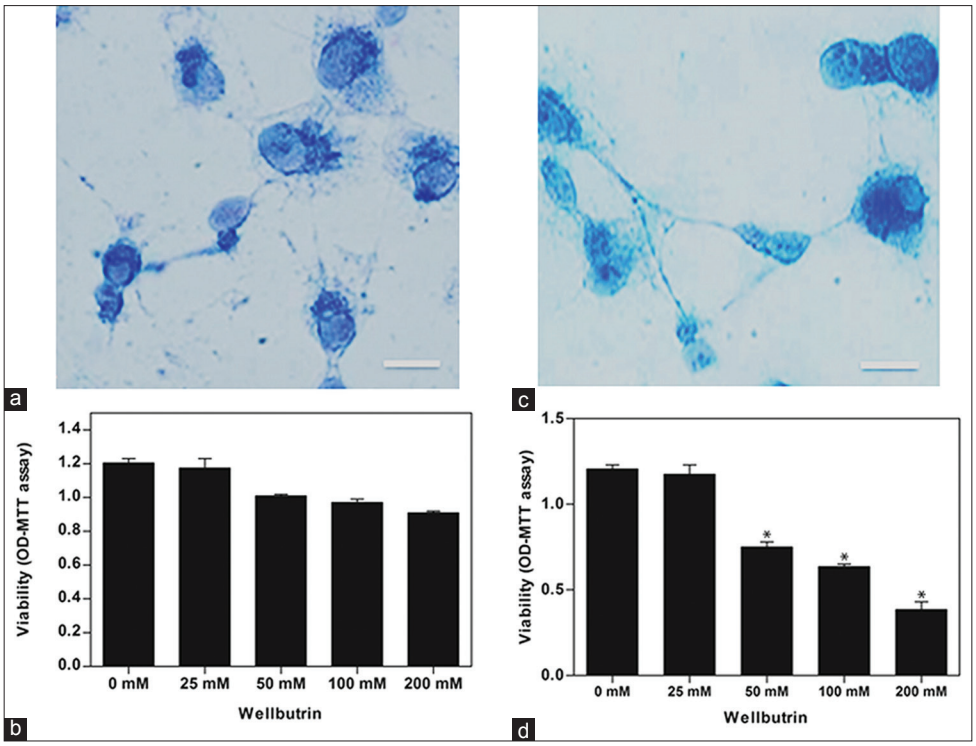


Figure 4: Loss of neuritis in human cortical neural 2 cells treated with wellbutrin. Cells were differentiated, treated, fixed and stained with Coomassie blue. The neurons were treated with different concentrations (a) at 24 h and (c) at 48 h, (b) and (d) are the data representing the percent cell viability at 24 and 48 h respectively. Scale bar=10 μm. *P<0.05

later times (48 h). This study illustrates that at later times, there was loss and disruption of all three major cytoskeletal filaments such as microfilaments and microtubules, and vimentin, which could explain the breakdown of the neurite morphology that was observed with the morphology studies at these time points. This modification in the cytoskeletal structures preceded cell death and loss in neurite morphology. Hence, this could be an early indication of impending cellular and morphological damage.[26]

Although, severe depression is clearly associated with several immunological changes, it still remains unclear that; how these drugs are associated with psychopharmacological treatments. There are very few reports available for the effects of anti-depressant drugs in human peripheral lymphocytes. In this study, an attempt has been made to assess the cytogenetic effects of WB in human peripheral lymphocytes. In a similar study conducted by Ronchetti *et al*. (2007)[27] in bupropion and paroxetin, no significant differences in cell viability were found. The major objective of our study was to find whether WB is capable of producing genotoxic or cytotoxic effects in human peripheral lymphocytes.

We report for the first time that WB is not capable of producing genotoxic effects in human peripheral lymphocytes. Previous studies observed a tendency of improvement in the immune response with administration of other dual action anti-depressants, with at least some serotonin action (mirtazapin).[28] Some other classes of anti-depressants exert inhibitory effects on the immune system. In summary, our systematic investigations on the induction and removal of DNA damage in WB-treated blood, in connection with the formation of SCEs and MN demonstrate induction of cytogenetic effects after exposure to WB under specific conditions. However, WB-mediated cytogenetic effects in blood cultures of humans are unlikely to occur, owing to lack of pre-requisite conditions.

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