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ESTIMATION OF CYTOTOXICITY AND GENOTOXICITY OF LONG ACTING BRONCHODILATOR: SALMETEROL XINAFOATE NANOPARTICLES (NANOTOXICITY STUDY)

RAGIA M. HEGAZY¹, EMAN M. FARUK²*, AMINA A. FARAG¹, LAMIAA M. SHAWKY²

¹Forensic Medicine & Clinical Toxicology Department, Benha Faculty of Medicine, Egypt
²Histology and Cell Biology Department, Benha Faculty of Medicine, Egypt

ABSTRACT

Nanoparticles (NPs) are now extensively used in different fields with inadequate knowledge on their toxicity. Salmeterol is a potent long acting-β agonist (LABA) used for the management of pulmonary disorders. Sothe present study was aimed to evaluating in vitro cytotoxicity and genotoxicity of nano salmeterol xinafoate nebulization. Blood samples from ten healthy volunteers were cultured and divided into two equal groups: control untreated group and other treated with a dose of nano salmeterol (50µg/ml) and incubated at 37°C for 24 hour then evaluated for local and systemic toxicity. Genotoxic study by using Comet assay and chromosomal karyotype was done. Morphological analysis of nano-sized salmeterol xinafoate particles was performed by Transmission Electron Microscopy (TEM) and Anderson Cascade Impactor (ACI) in which the average particle size was in the range of 300–500 nm. Our results showed that there was a non-significant decrease (p>0.05) in the level of antioxidant enzymes: ROS, GSH, SOD, and Catalase; while, there was significant increase (p<0.05) in the level of Lipid Peroxidation (LPO). The Comet assay indicated that cells treated with Salmeterol xinafoate NP induced non-significant increase in percentage tail DNA damage compared to control (p>0.05), Also, the chromosomal aberration was non-significant increase (p>0.05). So our study concluded that this dose of nano-salmeterol xinafoate NP nebulizer may be tolerated by asthmatic patients and further studies need in diseased patients and with different organs.

KEYWORDS: Salmeterol, Nanoparticles, TEM, Genotoxicity, Comet assay.

EMAN M. FARUK*
Histology and Cell Biology Department, Benha Faculty of Medicine, Egypt.
INTRODUCTION

Aerosol drug delivery has revolutionized the management of numerous respiratory diseases including asthma, obstructive airway diseases, interstitial lung diseases, pulmonary edema and cystic fibrosis.1,2 Aerosol therapy has numerous advantages over oral or intravenous drug delivery. It prevents first pass hepatic metabolism, requires low doses of inhaled drug and produces ultra-fine drug particles that can be deposited deeper in lung alveoli to cure pulmonary diseases. The commonly available device in the market for aerosols generation is nebulizer.3 Nebulizer can also be used to deliver high doses of a specific drug over a short period of time, such as during an acute exacerbation of asthma in emergency conditions.4 Salmeterol xinafoate is a potent and long acting β₂-adrenoceptor agonist with some anti-inflammatory activity and good specificity.5 Salmeterol causes the activation of adenylyl cyclase which in turn increases the level of intracellular 3', 5-cyclic adenosine monophosphate leading to the direct relaxation of airway smooth muscle cells and thus causes bronchodilatation.6,7 The long term action of salmeterol is due to its long carbon side-chain which is responsible for the lipophilicity and persistent retention of the molecule at the receptor site.8 The development of nano salmeterol nebulization solution is to maximize drug concentration with improved lung function while minimizing systemic toxicity supporting the necessity of alternative therapy to treat pulmonary diseases.9 The aerodynamic size of particles are the major determinant of aerosol deposition in the respiratory tract.10-11, so the inhaled drugs must ideally possess an aerodynamic diameter less than 5 μm to be delivered deep into lung for local therapy.12 Nowadays, there is increased exposure to nanoparticles among animals and humans due to the increase in their applications.13 Single cell gel electrophoresis or comet assay is a reliable assay to measure the genotoxicity of any compound. The comet assay is a simple, most reliable and inexpensive method to detect DNA damage in individual cells when treated with various chemicals, radiation, and drugs. This technique requires only few cells for obtaining the results within few hours.14 The present study was conducted to investigate the in-vitro cytotoxicity and genotoxicity of nano salmeterol xinafoate nebulization.

MATERIALS AND METHODS

Materials
Salmeterol xinafoate (Fluticasone) was procured from Sigma Chemical Company (St. Louis, MO, USA). Colchicine was purchased from Sigma chemical Co., St. Louis, USA. All other reagents were of analytical grade and were purchased from Merck Ltd. (Mumbai, India).

Study design
The study was designed with the following sequential components

Preparation of nano salmeterol xinafoate suspension
0.1 percent solution of Salmeterol xinafoate nebulization solution (SX-NS) was prepared in 0.9% normal saline containing 30% ethanol (7:3, v/v). The pH was adjusted at 7.4 and the formulation was passed through 0.22 micron filter. The formulation was prepared fresh daily before experiment and stored at 4°C. The average particle size was measured by using Anderson Cascade Impactor (Copley, UK) at the flow rate of 28.3 Lmin⁻¹. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of salmeterol xinafoate particles were also calculated.

Transmission electron microscopy (TEM)
Morphological analysis of nano-sized salmeterol xinafoate particles was performed by transmission electron microscopy (TEM, Philips CM-10, USA). The samples were prepared by placing a small drop of drug suspension on carbon coated copper grid covered with nitrocellulose and then negatively stained with 2% phosphotungstic acid (PTA). Digital micrographs of dried drug particles were taken by using TEM at the voltage of 300 kv.

Particle size distribution
Particle size distribution (PSD) of salmeterol xinafoate nebulization solution was also measured using photon correlation spectroscopy (Zetasizer, HAS 3000, Malvern instruments, UK).

Methods
Blood samples were taken from ten persons (aged 30 to 50) as normal volunteers according to UQU (Umm Alqura University). Institutional Ethics Committee for human studies (IECH). All donors participated in the study signed the written informed consent according to the approved procedure by UQU, Institutional Ethics Committee for human studies.

Culturing of human peripheral lymphocytes
Blood samples were drawn and the human lymphocytes were isolated from peripheral blood using the lymphocyte separation media, (HiSepTM,HiMedia, Mumbai) and 1x10⁶ cells was cultured using RPMI-1640 supplemented with 1% penicillin and 1% streptomycin. The 2% PHA was added to stimulate the cells and incubated for 24 hour in 5% CO₂ at 37°C.

Cellular viability by MTT assay
After 24 hour of incubation, the human lymphocyte cells were seeded into 100-well culture plates, 50–well culture plates treated with therapeutic dose of nano salmetrol (50µg/ml) and incubated at 37°C for 24 hour. After incubation, cells were treated with 5 mg/ml solution of MTT (3-(4,5- Dimethyl thiazol-2-yl)-2, 5-Diphenyl tetrazolium Bromide) at 37°C for 4 h in 5% CO₂ incubator. Then 50 µl of dimethyl sulfoxide (DMSO) was added to solubilize the formed formazan. The number of viable cells was read by Microplate Absorbance Reader (BioTek) at 532 nm. The human lymphocyte cells in the remaining culture plates untreated with NPs were taken as a control.

Cytotoxicity of the NPs in human lymphocytes
The cytotoxicity was assessed in human lymphocytes after 24 hour of NPs interaction using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), where cells (1.0×10⁶ cells/well) in 100 ml of DMEM were seeded in 96-well plates. The MTT assays have been
frequently used for determining the in vitro cytotoxicity of NPs in cell culture experiments.\textsuperscript{17}

**Oxidative stress (Cytotoxicity) in human lymphocytes**

Oxidative stress in human lymphocytes was estimated by the ROS generation to detect the intracellular reactive oxygen species (ROS). In 100 well culture plates, 1 × 10\(^5\) cells/ml seeded and incubated for 24 hrs at 37°C. After 24 hours different concentration of NPs (50, 75 and 100 µg/ml) was added and incubated for 24 hour in the 50-well culture plates. Then the cells were incubated at 37°C for 30 min with Dichloro-dihydro-fluorescein-diaceacetate (DCFH-DA).\textsuperscript{18}

**Lipid Peroxidation (LPO) and Antioxidant enzymes level**

The LPO was estimated by the malondialdehyde (MDA) formation, and 0.1 ml of cell suspension incubated at 37°C with 0.1ml of sodium phosphate buffer for 1 hour. The cells precipitated with 5% trichloroacetic acid and centrifuged at 2300g for 15 min to room temperature. Then 1% thiobarbituric acid was added and placed in boiling water for 15 min. After cooling at room temperature, the optical density (O.D) was read at 532nm using UV-Vis spectrophotometer (2201, Systronics) and represented as nmole of MDA/mg protein.\textsuperscript{19} The catalase activity in human lymphocytes was estimated by adding 0.1 ml of lymphocyte cells with 1 ml of H2O2 (30ml in phosphate buffer, pH 7.0) and 2 ml of 50 ml phosphate buffer (pH 7.0). The absorbance was measured at 240 nm for using UV-Vis Spectrophotometer (2201, Systronics).\textsuperscript{20} GSH level was estimated using Ellman's reagent. The assay mixture contained DTNB, phosphate buffer, and human lymphocyte cell extract. The absorbance was measured at 412 nm for the estimation of GSH.\textsuperscript{21} The SOD was done using the kit obtained from sigma Aldrich, USA. The absorbance was read at 440 nm by the decrease in colour development.

**Genotoxicity assessment**

The genotoxic potential of nano salmeterol NPs was assessed by the Comet assay as well as Chromosome Analysis (Karyotyping).

**Single Cell Gel Electrophoresis assay (Comet assay)**

25 µl of cell suspension was mixed with different concentration of NPs (50, 75 and 100 µg/ml) of 0.5% low melting temperature agarose (LMPA), and it was pre-coated with 1% normal-melting-temperature agarose. The slides were covered with a microscope cover slip and refrigerated for 5 min. The cover slip was removed, and a layer of 1% LMPA was added on to the slides. This was followed by immersion in ice-cold alkaline lysis solution(2.5mg sodium chloride, 10 ml Tris, 100mL Ethylenediaminetetraacetic acid (EDTA), 10% dimethylsulfoxide, 1% Triton X-100, pH 10.0) for at least 2hours. The slides were then incubated in ice-cold electrophoresis solution (0.3 mg sodium chloride, 1 ml EDTA, pH 13.0) for 20 min, followed by electrophoresis at 15V for 25 min. After electrophoresis, the slides were neutralized and stained by spreading ethidium bromide (20µg/ml). Analysis of comets was carried out using a fluorescence microscope (Leica DM-2500), and the percentage tail DNA was calculated based on (% tail DNA = 100 - % head DNA) for the quantification of DNA damage.\textsuperscript{22}

**Chromosome Analysis (Karyotyping)**

A chromosomal karyotype is used to detect chromosome abnormalities and thus used to diagnose genetic diseases. Chromosome aberrations were obtained from PHA-stimulated peripheral human lymphocytes. 23 Under aseptic conditions, 0.5 ml of venous blood was added and incubated at 37° C with 5 ml of RPMI-1640 medium, 1.2 ml of FBS and 0.3 ml of PHA. At the end of 1\(^{st}\) hour of incubation, the human lymphocytes were treated with 100µg/ml of NPs for 1 hour and centrifuged at 1000rpm for 5min. The cells were arrested by adding 0.01% of colchicine solution and then incubated for 5 min using 6 ml of the hypotonic solution; 6 ml of Carnoy’s fixative was added prior to slide preparations. The slides were prepared by gently placing cell suspension on a glass slide and dried on a hot plate. The chromosomes were treated with 0.25% trypsin that alters the structure of proteins followed by staining with Giemsa solution, and the slides were observed under microscope.

**Research ethics**

This experiment was accomplished under the approval of the state committee on human ethics, UQU, KSA and the recommendations of European Council Directive (86/609/EC), regarding the standards in the protection of human participating as a patient for research purposes were also followed.

**Statistical analysis**

The data obtained were statistically analyzed using SPSS software version (11.5). One-way ANOVA with Tukey's post-hoc test for comparison of different biochemical parameters was used for analysis. The results were expressed as mean ± standard deviation (SD). P<0.05 was considered as significant.

**RESULTS**

**Nanoparticles characterization**

The NPs were characterized by measuring the hydrodynamic size using DLS, but the shape and particle size of the salmeterol xinafoate NPs were studied by TEM.\textsuperscript{24}

**Aerosol size estimation**

Particle size of aerosolized salmeterol xinafoate analyzed by TEM photomicrograph of nano salmeterol xinafoate is shown in Figure 1(A) which suggested that salmeterol particles were round in shape with mean particle size of 300-500 nm. Figure 1(B).
Oxidative stress and antioxidant enzymes in human lymphocytes

A significant (p < 0.05) qualitative and quantitative concentration-dependent increase in % ROS generation was observed in the form of fluorescence on treatment with Salmeterol xinafoate NPs (Figure 2). The oxidative stress of Salmeterol xinafoate NPs in human lymphocytes showed significant increase in LPO level (P<0.05) meanwhile, there were non-significant decrease in catalase, GSH and SOD level (P>0.05) (Table 1).

Table 1

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>LPO (nmol MDA eq/g tissue)</th>
<th>GSH a (µg/mg protein)</th>
<th>SOD a (U/mg protein)</th>
<th>CAT a (µmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39.54±1.31</td>
<td>7.43±1.23</td>
<td>91.15±3.25</td>
<td>48.37±2.96</td>
</tr>
<tr>
<td>nanosalmetrol 50 µg/ml</td>
<td>40.07±0.39 a</td>
<td>5.93±5.4 a</td>
<td>89.23±6.07</td>
<td>47.83±1.69</td>
</tr>
<tr>
<td>nanosalmetrol 75 µg/ml</td>
<td>40.42±0.68 ab</td>
<td>6.29±4.09 a</td>
<td>90.26±1.84</td>
<td>48.41±4.81</td>
</tr>
<tr>
<td>Nanosalmetrol 100 µg/ml</td>
<td>42.13±5.23 abc</td>
<td>6.69±2.38 abc</td>
<td>91.09±2.75</td>
<td>49.09±4.25</td>
</tr>
</tbody>
</table>

a. significant differences compared to controls (P<0.007), b. significant differences compared to 50ug (P<0.02), c. significant differences compared to 75ug (P=0.02)

Figure 1

(A) TEM micrographs of salmeterol xinafoate-nebulization solution, (B) Nano-particle size of salmeterol xinafoate-nebulization 300-500 nm (TEM ×24000).

Figure 2

Photomicrographs showing the generation of intracellular reactive oxygen species (ROS) using DCFDA dye in human lymphocyte (Magnification X200). (A) Control cells and (B) Cells exposed to Salmeterol xinafoate NPs showing increase in fluorescence (Magnification X400).
Tail DNA migration
The obtained results from the comet assay indicated that cells treated with Salmeterol xinafoate NPs were able to cause non-significant increase in percentage (%) tail DNA damage compared to control (P>0.05) as shown in (Figure 3).

![Figure 3](image)

**Figure 3**
Induction of DNA damage by Salmeterol xinafoate NP in human whole blood cultures. Data considered significant if P < 0.05.

Chromosomal aberrations
The minimum of 50 metaphases per sample scored for the chromosome aberration assay. When compared to control an insignificant increase (P>0.05) in chromosomal aberrations was observed when exposed Salmeterol xinafoate NPs (table 2) and (Figure 4).

![Figure 4](image)

**Figure 4**
Chromosomal aberration in metaphase chromosomes of human lymphocytes. A) Control; B) Chromosome break (arrow) C) Chromosome deletion (arrow) D) Chromosome deletion gap. There are non-significance difference between Salmeterol xinafoate NPs versus control: P >0.05 in Chromosomal aberration.

<table>
<thead>
<tr>
<th>Salmeteol xinafoate NPs(µg/mL)</th>
<th>Chromosome number Mean ± SE</th>
<th>deletion</th>
<th>Breaks</th>
<th>Gaps</th>
<th>Aberrant Cell Damage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>46±0.12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>75</td>
<td>46±0.24</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2%</td>
</tr>
<tr>
<td>100</td>
<td>46±0.15</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>3%</td>
</tr>
</tbody>
</table>

Aberrations/number of metaphases analysed × 100.
DISCUSSION

The use of NPs has increased in various fields, and it therefore the need to investigate the toxic effects of the NPs on human health has become very essential. The NPs toxicity is mainly due to the structure, solubility, shape and surface/mass ratio. Nano salmeterol xinafoate nebulization is now used in treatment of chronic pulmonary disease. Several studies have been done to assess their toxicity in mammalian cells. The present study was aimed at studying the effect of the in vitro cytotoxicity and genotoxicity of nano salmeterol xinafoate nebulization. Nano particles have greater efficacy in comparison to micronized drug due to their ability to penetrate deeper into the lung parenchyma. TEM analysis of salmeterol particles also confirmed that the particles were round in shape and the aerosol size was in nano range having mean particles size 300-500 nm. A key component of the present formulation of salmeterol xinafoate nebulization solution for inhalation is ethyl alcohol (ethanol). Ethyl alcohol has been used in a variety of pharmaceutical formulations such as Azmacort, Respithaler and Bronchometer etc. Nano size of salmeterol drug particles was achieved by using ethanol in the formulation which had inherent antifoaming nature. Ethanol lowers the surface tension and helps in drying of vaporized particles thus increased the drug out-put by 60-70% and lowers the nebulization time by 30-50%. Ethanol improved the drug aerosol deposition deeper into the lung and lowers the chances of being expelled out due to physiological process. The cytotoxicity observed with NPs might be due to the pro-inflammatory effects through ROS mediated mechanism. Oxidative stress occurs when ROS disturbs the balance between oxidative and antioxidant defence levels. The ROS was due to the superoxide radical, hydrogen peroxide (H2O2), and the hydroxyl radical which causes DNA damage, and apoptotic cell death. The NPs showed toxic effects on the mitochondrial function leading to the generation of ROS and alteration in the antioxidant level of human lymphocytes. In the present study the Salmeterol xinafoate NPs were found to produce non-significant oxidative damage to blood lymphocytes beside affecting the DNA. In many researches, the internalisation and easy penetration of NPs into the cells was found to be the main reason regarding the size-dependant toxic effects of NPs. The toxicity in human lymphocytes was mainly due to the pathway that involves the endocytosis of NPs agglomerate. The oxidative stress disturbs the cellular macromolecules such as lipids, proteins, and DNA when exposed to NPs. The DNA damage occurs mainly due to mutations, deletions, cross-linking and adds formation leading to apoptosis. The caspases play a significant role in the initiation and execution of apoptosis causing cellular DNA damage by NPs in human lymphocytes. The Salmeterol xinafoate NPs caused chromosomal DNA fragmentation and nuclear condensation in human lymphocytes. In recent research, the genotoxicity of NPs was due to chromatin modification, ROS induction and DNA liberation from the lysozyme. The cellular production of ROS can cause damage to cellular macromolecules such as DNA. The chromosomal aberration is a genetic marker to study the genotoxicity risk of human leukocytes. Alteration in the chromosome could be any changes in the number of chromosomes or the structure of chromosome. The chromosomal aberrations like break and deletion observed after exposure to salmeterol xinafoate NPs are non-significant. Chromosomal aberrations were believed to be an important aspect resulting in cell cycle arrest, and it was an important marker for cancer risk. In other researches, the penetration of ions into the cell causes crosslink in the DNA, sister chromatid exchange and mutations leading to cancer risk when exposed to metal oxide NPs. The results have proven that the salmeterol xinafoate NPs were able to cause minor DNA damage and limited chromosomal aberration in human lymphocytes.

CONCLUSION

To conclude, the present inhalational formulation of salmeterol xinafoate offers great promises for pulmonary delivery of drug which inherits good in-vitro aerosolization in nano size that settle deeply into the lung parenchyma and alveolar region. The results highlight the safety of salmeterol xinafoate in nano-size formulation, establishing that it can be used to treat pulmonary diseasted patients. But we need further study to ensure that nano-salmeterol xinafoate safer in other organs.

CONFLICT OF INTEREST

Conflict of interest declared none.

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