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Comparison of the cytotoxic, genotoxic and apoptotic effects of Sugammadex and Neostigmine on human embryonic renal cell (HEK-293)

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Abstract: Acetylcholinesterase inhibitors, including Neostigmine, have been used to reverse neuromuscular blockage for many years. Sugammadex reverses this blockage using its gamma cyclodextrin ring, a mechanism that differs from that of cholinesterases and so circumvents the side effects of Neostigmine. Although the superiority of Sugammadex to Neostigmine has been outlined in several clinical studies, to our knowledge, there is not any research into cell culture that compares the cytotoxic, genotoxic and apoptotic effects of the two drugs. Hence, this is the first study to compare the cytotoxic, genotoxic and apoptotic effects of different dosages of both drugs on human embryonic renal (HEK-293) cells. In this study, the cytotoxicity, genotoxicity and apoptotic effects of Sugammadex and Neostigmine on HEK-293 cells were analyzed with using the MTT, Comet Assay and Flow Cytometric Annexin-V methods, respectively. The results demonstrate that Neostigmine at 50, 100, 250, and 500 µg/mL is more cytotoxic than equivalent dosages of Sugammadex. Neostigmine at 500 and 1000 µg/mL was found to be more genotoxic, and Neostigmine at 500 µg/mL had a statistically higher risk of causing apoptosis and necrosis than Sugammadex ($p<0.05$). Neostigmine administered in-vitro in the same doses as Sugammadex had greater cytotoxic, genotoxic and apoptotic effects on HEK-293 cells.

Key words: Anesthesia; Apoptosis; Cytotoxicity; Genotoxicity; HEK293 cells; Neostigmine; Sugammadex.

Introduction

Neuromuscular blocking agents are often used intraoperatively to facilitate tracheal intubation and improve surgical conditions. Acetylcholinesterase inhibitors, including Neostigmine, are conventionally used to reverse the non-depolarizing neuromuscular blockage agents (1). Anticholinesterase agents are used in combination with anticholinergics including atropine and glycopyrrolate to prevent potential muscarinic side effects on the cardiovascular, gastrointestinal and pulmonary systems. However, these agents might, in turn, cause nausea,vomiting, hypersecretion, cardiac rhythm abnormalities and bronchospasm (2).

Sugammadex is a modified gamma-cyclodextrin molecule that has recently entered practice in clinical neuromuscular pharmacology. Sugammadex encapsulates the steroid neuromuscular blockage agents (Rocuronium and Vecuronium) and detaches these from nicotinic acetylcholine receptors just like a synthetic receptor (3). Sugammadex is a biologically inactive, well tolerated and reliable agent and does not lead to the cardiovascular or hemodynamic side effects that are caused by Neostigmine and the combined anticholinergics. Sugammadex resolves the neuromuscular blockage rapidly and reliably, and the molecule also decreases the postoperative residual blockage risk and incidences of severe postoperative pulmonary complication (4,5).

A preliminary literature survey revealed that although the superiority of Sugammadex to Neostigmine has been outlined in several clinical studies, there have been to date no cell culture studies that compared the cytotoxic, genotoxic and apoptotic effects of both drugs. Accordingly, to our knowledge, this is the first study in the literature that compared the cytotoxic, genotoxic and apoptotic effects of Sugammadex and Neostigmine on human embryonic cells (HEK 293).

Materials and Methods

Chemicals

Fetal Bovine Serum (FBS), Streptomycin/peniciline, Trypsin and Dulbecco's modified Eagle Medium (DMEM: F12) were obtained from Sigma–Aldrich (Seelze, Germany), while Sugammadex (Bridion®; N.V. Organon, Holland) and Neostigmine (Neostigmine®; Adeka, Turkey) were procured from pharmaceuticals.

Samples

Sugammadex and Neostigmine were both dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution of 2 mg/ml. The solution was diluted with a DMEM:F12 medium and dilution continued until the DMSO concentration of the drug solutions was <1%. Upon confirmation that such a concentration of DMSO and a non-serum containing medium does not cause DNA injury, experiments were initiated with the other freshly prepared reactive, all of which were prepared freshly before each experiment.

Cell culture

The study cell line (HEK-293) was attained from the American-Type Cell Culture Collection (ATCC). The HEK-293 cell line was cultured in a 10% FBS and 1% streptomycin/penicillin with added DMEM:F-12 medium at 5% CO_2 containing a 37ºC humidified incubator. The medium was changed at one-day intervals and upon the achievement of 100% confluence, the flask surface was removed using Trypsin and cells were used to constitute the experiment kits. The number of live cells was determined through a trypan blue exclusion test.

Cytotoxicity (MTT) Assay

The cytotoxicity of Sugammadex and Neostigmine were established via a 3-(4,5-dimethylimidazole-2-il)-2,5-diphenyltetrazolium bromide (MTT) test, which relies on the cleavage of tetrazolium salt by mitochondrial succinate-tetrazolium reductase in vivo (6). HEK-293 cells were inoculated into a 96-well petri dish (10^4 cell/ml) and incubated at 37°C in 5% CO_2 for 24 hours, after which, the cells were exposed to Sugammadex and Neostigmine at 0, 5, 10, 25, 50, 100, 250 and 500 µM dosages for 24 hours. At the end of 24 hours, the medium was removed, the cells were irrigated with phosphate-buffered saline (PBS), and MTT stain (5mg/mL) was added to the medium. The medium was removed after four hours of incubation. Finally, 200 µl DMSO was added and stirred for 10 min to allow the measurements to be taken using a spectrophotometer device (Spektramax M5) at 570 and 630 nm. Cytotoxicity was expressed as the mean percent increase (mean ± standard deviation) compared to the controls, which were unexposed to the specified substances. The control values were set as 0 percent cytotoxicity, and the concentration (IC50) that resulted in 50% inhibition when compared to the unprocessed controls was calculated.

Single Cell Gel Electrophoresis Assay (Comet assay)

The genotoxic effects of Sugammadex and Neostigmine on HEK-293 were evaluated using a modified form of the alkaline single cell gel electrophoresis assay (comet assay) developed by Singh et al (7,8). HEK-293 cells were inoculated into the six-well petri dish (approximately $2x10^5$cells/cell) and incubated in 5% CO_2 at 37°C for 24 hours to determine the genotoxic potential of Sugammadex and Neostigmine. At the end of 24 hours, varying concentrations of Sugammadex and Neostigmine (0, 100, 250, 500, 1000 ug /ml 1% DMSO) were added to the culture, which was incubated for another 24 hours at 37°C. DMSO (1%) was used as the negative control, and 50 mmol/L H_2O_2 was used as the positive control. The comet tail formation around the nucleus was examined to determine the nuclear DNA injury, for which 100 nuclei were selected randomly and examined manually under the microscope. The injury was scored on a scale from 0 to 4 (0: no injury, 4: severe injury), while the total visual comet assay score characterizing the degree of DNA injury in all study groups was the sum of five distinct comet assay scores. Accordingly, the total visual score ranged between 0 (no injury) and 400 (maximum injury) arbitrary units (AU). The results of the triple tests were expressed as arbitrary units, and all assays were repeated three times each.

Annexin V-FITC Apoptosis Assay

The Muse Annexin V & Deal Cell Assay Kit protocol was administered before using the Muse cell analyzer (Millipore) to determine whether the activation of the apoptotic pathway or cellular necrosis was behind the potential cytotoxic effects of Sugammadex and Neostigmine. Annexin-V binds FITC used in apoptosis analyses and conjugates Annexin-V lectin with phosphatidylserine phospholipids on the outer cell membrane of apoptotic cells. FITC causes the fluorescent radiation of Annexin-V bound cells (FL1 detector; excitation = 488nm, emission=535nm), and this fluorescent radiation of the cells was determined from the FL1 detector in flow cytometry. Cells were classified according to the degree of radiation and plotted on a diagram, while dead cells were identified using a fluorescent PI stain (FL2 detector, excitation= 488nm, emission=562-588nm) that binds nucleic acids. PI permeates the injured cell membrane of necrotic cells to stain their DNA, and the fluorescent radiation of cells with stained DNA is then identified with the FL2 detector on flow cytometry. The cells were classified according to the degree of radiation and plotted on a diagram. The apoptotic effect of a 500 µg/mL dosage of Sugammadex and Neostigmine, whereby the agents exerted high cytotoxicity on HEK-293, was investigated in this way.

The HEK-293 cells were inoculated into the six-well petri dish ($1x10^6$ cells/ml) and incubated for another 24 hours at 37°C. At the end of 24 hours, the specified 500 µg/mL dosage of Sugammadex and Neostigmine were added, and incubation was continued for another 24 hours at 37°C. The next day, the cells were prepared using the Muse Annexin V & Dead Cell Assay kit protocol and evaluated using the proper program.

Statistical analysis

The results are presented as the mean ± standard deviation (SD) of the three repetitions. All data from experiments were analyzed for statistical significance using an analysis of variance (One-way ANOVA), and a p-value of <0.05 was considered statistically significant. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 20.0 (IBM Corp., NY, USA).

Results

The Cytotoxic Effects of Sugammadex and Neostigmine on HEK-293

The effects of Sugammadex and Neostigmine on the viability of HEK-293 cells were determined using the MTT method, and it was found that the percentage of cytotoxic effect increased with higher concentrations and Neostigmine exerted more cytotoxic effects on HEK-293 cell series than Sugammadex. Neostigmine was observed to be statistically and significantly more cytotoxic than Sugammadex, particularly in 50, 100, 250, and 500 µg/mL concentrations ($p<0.05$) (Figure 1). The IC_{50} of Neostigmine on HEK-293 cells was 657.8 ug/mL, and 1870.2 µg/mL for Sugammadex.

The genotoxic effect of Sugammadex and Neostigmine on HEK-293

The genotoxic effects of Sugammadex and Neo-

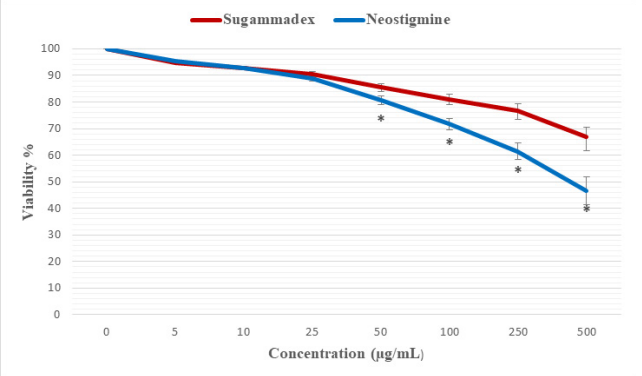

Figure 1. The effects of Sugammadex and Neostigmine on HEK-293 viability over a 24-hour period.
* : significant difference between two drugs at the same concentration ($p<0.05$) Results are presented as the mean ± standard deviation of the three independent experiments.

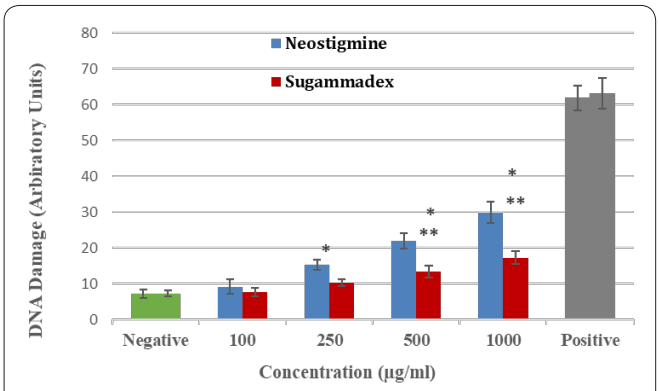

Figure 2. DNA damage rate by the mean of comet formation in HEK-293 cells treated with Sugammadex and Neostigmine.
* :Concentrations with significant differences compared to the negative control. ($p<0.05$) ** : Significant difference between two drugs at the same concentration. ($p<0.05$). Results are presented as the mean ± standard deviation of the three independent experiments.

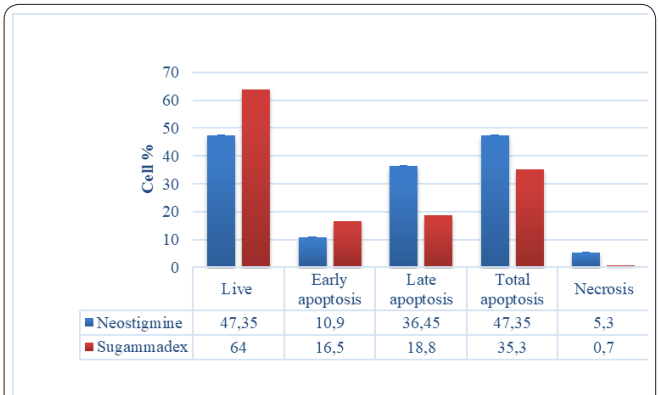

Figure 3. Induction of apoptosis in HEK-293 cells by Sugammadex and Neostigmine. The apoptosis ratio analyzed by flow cytometry. Data analyses of HEK-293 cells of Annexin V/PI flow Cytometry,

stigmine on HEK-293 were compared using the Comet Assay method. Significant DNA injury occurred when cells were exposed to increasing dosages of either drug when compared to the negative controls at 250, 500 and 1000 µg/ml dosages. The level of DNA injury was significantly lower with both drugs at all dosages when compared to the positive control, although Neostigmine caused greater DNA injury than Sugammadex at all dosages. At doses of 500 and 1000 µg/ml, Neostigmine caused significantly greater DNA injury than Sugammadex ($p<0.05$) (Figure 2).

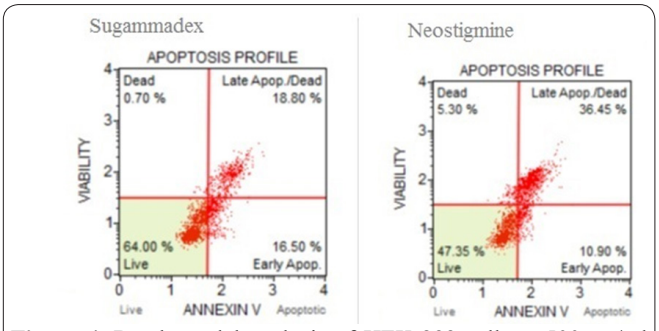

Figure 4. Death model analysis of HEK-293 cells at 500 µg/ml concentration of Sugammadex and Neostigmine.

Flow cytometric annexin-V apoptosis/necrosis analysis.

An investigation of the apoptotic effects of Neostigmine and Sugammadex on HEK-293 cells showed that both drugs led to an apoptotic effect at 500 µg/ml. Neostigmine-exposed cells led to early apoptosis in 10.90%, late apoptosis in 36.45% and necrosis in 5.30%, while Sugammadex-exposed cells led to early apoptosis in 16.5%, late apoptosis in 18.80% and necrosis in 0.70%. The apoptosis analysis demonstrated that Neostigmine exerted greater apoptotic effects than Sugammadex at a 500 µg/ml dosage ($p<0.05$) (Figure 3,4).

Discussion

The objective of this study was to compare the cytotoxicity, genotoxicity and apoptosis features of Sugammadex and Neostigmine in-vitro. Although Sugammadex has been shown to be superior to Neostigmine in several clinical studies, this was the first study to compare the in-vitro effects of the two drugs. The results of this study show that Sugammadex is superior to Neostigmine in a dosage-dependent manner regarding the cytotoxicity, genotoxicity and apoptosis effects on HEK-293 cell series.

The lipophilic gamma cyclodextrin structure of Sugammadex comprises rigid glucose molecules in a ring shape. Steroid neuromuscular blockage agents (Rocuronium, Vecuronium) permeate the cyclodextrin ring of Sugammadex to form a complex and lead to ineffective Rocuronium on the nicotinic receptors and the rapid termination of the neuromuscular blockage (9). The cyclodextrin structure has high selectivity towards steroid neuromuscular blockers, and this feature has placed Sugammadex as the primary representative of the "selective relaxant binding agents" drug class (10).

Sugammadex reverses neuromuscular blockage independently of acetylcholinesterase, and so prevents such adverse effects as autonomic instability of the anticholinesterases like Neostigmine and combined antimuscarinic agents. Sugammadex does not bind to plasma proteins or erythrocytes and is distributed throughout the extracellular fluid in the body reaching to volume of 11-14 liters in an adult. The drug is biologically inactive and generally does not bind to plasma proteins (11).

The recommended dose of Sugammadex for reversal of Rocuronium and Vecuronium-induced neuromuscular blockade is 2-4 mg/kg. If there is a clinical need to reverse Rocuronium induced neuromuscular blockade

within 3 minutes after administration of a single dose of Rocuronium, a Sugammadex dose of 16 mg/kg is recommended (12).

In addition, the recommended dose of neostigmine for reversing neuromuscular blockade is 0.03 - 0.07 mg/kg. The maximum effective dose of Neostigmine is in the range of 0.06 to 0.08 mg/kg, and the recommended dose for blockade reversal in pediatric patients is 0.02 to 0.06 mg/kg when combined with 0.02 mg/kg atropine (13).

Recently, there have been many clinical studies comparing Sugammadex with Neostigmine (14-17). Compared with Neostigmine or placebo, Sugammadex reverses Rocuronium-induced neuromuscular blockade rapidly and safely in adults (14) and pediatric patients (15). According to the review of 41 studies on 4206 patients regarding the clinical effects of both drugs, the researchers found significantly fewer adverse events in the Sugammadex group compared with the Neostigmine group (16).Results from an another meta-analysis suggest that; Sugammadex accelerates postoperative discharge of patients after general anesthesia compared with Neostigmine (17).

The high cost of Sugammadex has so far limited its routine use. Sugammadex promotes a rapid turnover of patients in the operating room, which is cost-effective but limits the disadvantage of its high cost. Through a rapid, predictable, and safe reversal of the rocuronium-induced neuromuscular block, Sugammadex minimizes the risk of postoperative residual curarisation and its consequences (18,19).

Cyclodextrins are in frequent use in the medical and food sectors. Specifically, cyclodextrin molecules are used to convert lipophilic agents into hydrophilic forms. Some cyclodextrins may alter the receptor stability and the structure of the lipid body on membranes, which in turn can affect receptor functions and cause a release of cholesterol from the cell membrane. The glucose rings on cyclodextrins (D-glucopyranose units) have a three-dimensional structure, and this ring-shaped structure contains a conical hydrophobic space on its inner side and a hydrophyllic structure on the outside. They are labeled according to the number of glucose rings, with alpha having six rings, beta having seven and gamma having eight. Narrow and larger openings can be found on the primary and secondary faces, and the negative hydroxyl groups on the primary and secondary faces render the molecule water soluble. Carbon atoms within the alpha 1-4 bonds provide a lipophilic gap and allow the water-soluble molecule to surround a lipophilic nucleus. This structure covers the properly sized lipophilic drugs and enhances water solubility, and non-covalent thermodynamic interactions lead to inclusion complexes (molecular encapsulation). The size of the gap, at 0.8 nm, is greater in gamma-cyclodextrins than in alphas and betas. Thermodynamic, Van der Waals, hydrophobic, hydrogen and charge transfer interactions contribute to the formation of inclusion complexes (host-guest complexes) (20). Inclusion complexes encapsulate a lipophilic molecule, and some studies have demonstrated that substances with different cyclodextrin structures are protective against hypoxia (21,22).

To our knowledge, there is not any study that is comparative in-vitro studies of the joint effects of Sugammadex and Neostigmine, although many different studies have utilized the experimental ischemia/reperfusion model of both drugs (22,23).

A study of rats with cerebral ischemia/reperfusion injury found that Sugammadex at 16 mg/kg and 100 mg/kg dosages have neuroprotective effects and can provide protection against cerebral ischemia, and researchers have suggested that this effect probably stems from the gamma cyclodextrin ring of Sugammadex (22).

In another experimental study, researchers exposed NSC-34 neuronal cells to 30 μM Sugammadex. They found the amplitude and gating of delayed-rectifier K+ current may be modified and these actions might significantly contribute to functional activities of motor neurons (24).

A similar cerebral ischemia/reperfusion study investigated the protective effects of Neostigmine and Anisodamine, and the authors concluded that a combination of Neostigmine and Anisodamine reduced apoptosis by inhibiting the mitochondrial pathway in cerebral ischemia and that the α7 nicotinic acetylcholine receptor pathway played an important role in achieving this goal (23). In an in-vivo experimental study investigating the effects of neostigmine on mouse bone marrow, Neostigmine was found ineffective on cell division and not genotoxic in somatic tissue (25). However, an in-vitro study of the effects of Sugammadex on neuron cells revealed that cell death through apoptosis occurred at 24 hours in cells exposed to 75 μg/ml Sugammadex. Sugammadex increases the expression of the monoclonal cytochrome C-protein (CytC), apoptosis inducing factor and CASP-3 proteins, and it has been suggested that Sugammadex alters cholesterol hemostasis in response to oxidative stress, and thus leads to apoptotic activation in neuronal cells. The authors emphasized that the level of resistance or susceptibility to oxidative stress may depend on several factors, among which can be listed anatomical regions of the brain, neuronal cell types, potential neuron-astrocyte interactions or pathological conditions, including inflammation (23,26).

Although interesting, in-vitro results do not always correlate with in vivo results for a few reasons. In-vitro studies employ a simpler system to the human organism, while in vivo cells do not enter into a static tampon, and their extracellular drug concentrations are dependent on absorption, distribution, clearance and protein binding (27). We believe this is the underlying reason behind Sugammadex's exhibiting of neurotoxicity in-vitro cell cultures, despite its proven protective effects against cerebral ischemia in an experimental animal model.

Although the clinical effects of Sugammadex and Neostigmine have been compared on several occasions, the most significant characteristic of our study has been the in-vitro comparison of their effects on the same cell series. The study dosages were examined independently of clinical dosages, and the objective of the cytotoxicity study was to investigate the effects of both drugs used at the same dosages.

The findings indicate that Sugammadex provides better results than Neostigmine regarding its cytotoxicity, genotoxicity and apoptosis effects on HEK-293. Neostigmine at 50, 100, 250, and 500 μg/mL was found to be more cytotoxic when compared to equivalent dosages of Sugammadex, and Neostigmine at 500 and 1000

μg/mL was more genotoxic ($p<0.05$). Neostigmine at 500 μg/mL presented a significantly higher risk of causing apoptosis and necrosis when compared to Sugammadex ($p<0.05$).

In this first study that compared the in-vitro effects of Sugammadex and Neostigmine when used for the same clinical purpose, despite different mechanisms of action, we found that Sugammadex led to less cell injury than Neostigmine within the same dose ranges. Further in-vitro comparison studies that involve other cell series and at different clinical dosages would be beneficial to provide valuable insights into the literature.

Conflicting Interest Statement

There are no conflicts of interest.

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