XANTHAN-CHITOSAN COMPLEX AS A POTENTIAL PROTECTOR AGAINST INJURIOUS EFFECTS OF NEOMYCIN

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The aim of this paper was to achieve neomycin sulphate (Ne) inclusion using a hydrophilic xanthan-chitosan polyionic complex (Xa-Cs) to develop an oral controlled release drug delivery system and a potential protector against injurious effect of Ne. The obtained samples were characterized by FT-IR spectroscopy, SEM microscopy and the study of the swelling behavior in simulated gastric fluid (pH = 2.0) and intestinal fluid (pH = 7.2). An in vivo study was conducted on healthy wistar rats. The drug dose chosen for *in vivo* study was 50mg Ne/100g body weight or an equivalent dose in complex form. The treatment with Xa-Cs-Ne ameliorated the biochemical parameters (creatinine, urea, serum uric acid and C-reactive protein levels) and histological changes of colon due to drug toxicity.

Keywords: xanthan, chitosan, neomycin, protector

INTRODUCTION

Neomycin (Ne) is an aminoglycoside antibiotic produced from the fermentation of the actinomycete *Streptomyces fradiae*, which shows antibacterial activity against both gram-positive and gram-negative bacteria.[1] It is used in creams, ointments, solutions and tablets.[2] In therapy, Ne is used in its sulphate form, which consists of a complex of three aminoglycosides: neomycins A, B and C, with neomycin B in the highest proportion.[1,3] The administration has been restricted due to its toxicity. The complications include severe hearing loss, renal toxicity and mal absorption syndrome.[4] Ne reduces the population of aerobic intestinal bacteria, leading to increased intestinal permeability and therefore to adverse effects on the physiology, morphology, and histology of the gastrointestinal tract (GIT).[5]

Chitosan (Cs), the main derivative of chitin, is a heteropolymer consisting of linked D-glucosamine or N-acetyl-D glucosamine residues. The primary amine at the C-2 position of the D-glucosamine residues allows specific chemical reactions and confers important functional properties to chitosan.[6] It is a non-toxic natural polycationic polymer that is degraded by the microflora in the colon.[7]It shows unique properties, including bioactivity, biocompatibility and biodegradability.[8] Cs is used as an antibacterial, anticancerous, anticoagulant, antifungal, immunostimulant, wound healing, antiinflammatory, fat blocking, kidney function modulator, antiviral and antioxidant agent.[9]

Xanthan gum (Xa) is a high-molecular weight polysaccharide containing D-glucose, D-mannose and D-glucuronic acid units, produced by the fermentation of *Xanthomonas campestris*.[10] This is one of the most extensively investigated polysaccharides with respect to biocompatibility, stability and safety.[11]Xa is non-toxic and is totally degraded by bacteria resident in the colon.[12,13] It is an excellent suspending, stabilizing, thickening and emulsifying agent, and for these reasons it is widely used in the pharmaceutical industry.[14]

Hydrogels based on Cs are frequently used in pharmaceutical applications, such as sustained-release matrix tablet of propranolol hydrochloride, floating and mucoadhesive beads for controlled release of glipizide, controlled release formulation of theophylline, bioadesive controlled release matrix of metronidazole, oral controlled release

matrix of ambroxol hydrochloride.[15,16-19]

The Xa-Cs polyelectrolyte complex was used for the encapsulation or the entrapment of the *Lactobacillus acidophilus*, a probiotic enzyme (firefly luciferase), secoisolariciresinol antioxidant and for the preparation of nanoparticles based on the insulin hormone.[20-22]

Based on previous reports, the main purpose of this study was to entrap Ne into a Xa-Cs complex and to evaluate the protective influence of the polymer matrix against the injurious effects of the raw drug.

EXPERIMENTAL

Materials

Cs (Mw = 94.8 kDa, with a polydispersity index of 3.26 and deacetylation degree of 79.7%) was purchased from Vanson, Inc. (Redmond, W.A. USA). Xa was obtained from BioChemika, Australia (degree of substitution per side chain of 0.73 and 0.75 for acetate and pyruvate groups, respectively, as determined by proton 1H NMR). Ne (Hebei Rongqing Biotechnology Co.Ltd.) was a kind gift sample. All other chemicals and reagents used were of analytical and pharmaceutical grade.

Methods

A Xa solution (0.65%, w/v) and a Cs solution (0.65%, w/v) were prepared. 2200 ml of the Xa degassed solution was added dropwise using a syringe needle (1.1 mm diameter) controlled by a peristaltic pump (flow rate 1.5 ml/min), to 1000 ml Cs solution. The above solution was stirred continuously for maturation, using a magnetic stirrer, for 30 minutes, at room temperature. The hydrogel capsules were filtered, then washed with distilled water several times, until a neutral pH was achieved and freeze-dried (Alpha 1-4 LSC, Christ, Germany). The method for loading the obtained Xa-Cs complex with Ne consisted in the diffusion of a drug solution into the hydrogel network. 1 g lyophilized capsules were suspended into 10 mL Ne solution under mild stirring. The total diffusion of the drug was reached after 10 min. The obtained Xa-Cs-Ne complex was then freeze-dried.

FT-IR Spectroscopy

Infrared transmission spectra were obtained using a BOMEM MB 104 FTIR spectrophotometer (Canada). The samples were dispersed in potassium chloride (6% w/w), ground to fine powder using an agate mortar and then compressed into a KBr disc at 10000 psi, which was then scanned with a resolution of 4 cm^{-1} in the wavenumber range of 400-4000 cm^{-1}.

SEM

A scanning electron microscope (Vega II LSH with accelerating voltage of 30 kV, Tescan Company) was used to examine the morphology of the Xa-Cs and Xa-Cs-Ne complex. The samples were attached to aluminum holders using a silver based adhesive. Sample conductivity was achieved by sputter coating with a 15 nm layer of gold.

Determination of the swelling degree

Swelling studies were carried out by direct immersion of the freeze-dried Xa-Cs-Ne complex in 200 mL hydrochloric acid solution (pH 2.0) or 200 mL phosphate buffer solution (pH 7.4). Then the samples were periodically removed from the solutions, gently wiped with a soft tissue to remove the excess surface solution, weighed and then carefully placed back into the vessel as quickly as possible. This experiment was continued until the weight of the sample appeared to be constant. The reported weight of each sample was an average value of three measurements. The swelling degree (SD) was calculated according to the equation:

$$SD\% = \frac{W_s - W_d}{W_d} \times 100 \quad (1)$$

where W_s and W_d represent the weight of swollen and dry Xa-Cs-Ne samples, respectively.

In vivo assay

The *in vivo* experimental protocol employed in the present study was strictly in accordance with the European Community guidelines regarding ethics and approved by Apollonia University of Iasi. The animal breeding facility of the Cantacuzino Institute, Bucharest, Romania, supplied adult male Wistar rats with an average weight of 265 ± 15 g. Rats were fed daily with a standard diet of known composition and water *ad libitum*. The animals were housed in plastic well-aerated cages at normal atmospheric temperature (22 ± 3°C), relative humidity of 50±5% and were exposed to normal cycles of day and night.

Nephrotoxicity was induced by oral gavage, once daily, for a period of 14 consecutive days. After an acclimatization period of 7 days, the animals were randomly divided into three experimental groups of six rat each as follows: (I)) control, (II) Ne and (III) Xa-Cs-Ne. Group I received only sterile water, group II received Ne in sterile water, at a dose of 50 mg/100 g body weight and group III, Xa-Cs-Ne containing an equivalent dose of Ne. Body weight was measured again on day 10. After this treatment regimen, the rats were anaesthetized and blood was collected via retro-orbital bleeding for determination of biochemical parameters (creatinine, urea, uric acid and C-reactive protein levels). After collection of the samples, the animals were sacrificed, and organs were removed and stored in buffered formaldehyde, for histological examination. The kidneys were sectioned longitudinally and the colon was rolled to allow microscopic analysis of the entire colon.

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM). The differences among the treated

groups were analyzed by one-way ANOVA, followed by Tukey's test. The significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

FTIR spectroscopy

Fig. 1 depicts the FTIR spectra of raw Ne, Xa-Cs hydrogel and Xa-Cs-Ne complex. The spectrum for Ne shows a characteristic peak at 3267 cm^{-1}, which corresponds to -OH vibrations conjugated with -NH and aromatic -CH, a band at 1339 cm^{-1} attributed to C-N stretching, a band at 1070 cm^{-1} attributed to C-O stretching and another band around 1524 cm^{-1} representing C-C stretching vibrations of aminoglycoside.[23]The bands around 1628 and 1121 cm^{-1}correspond to the N-H bending and ether frequencies, respectively. A band near 619 cm^{-1} might be assigned to the N-H out of plane bending of Ne.[24]In the FTIR spectrum of Xa-Cs (Fig. 1b), the bands attributed to Xa and Cs can be observed. Xa shows distinctive bands at 3427 cm^{-1}, 2922 cm^{-1} and 1065 cm^{-1}, which are assigned to O–H stretching, C–H stretching vibrations and –C=O of pyruvate group in Xa. Cs from the complex exhibits distinctive bands at 1614 cm^{-1} (NH_2).[15]The peak corresponding to amide I from raw Cs (1654 cm^{-1}) is not observed because of the intermolecular interactions between Xa and Cs. A quite intense band appears at 1408 cm^{-1}, indicating that the NH_3^+ group of Cs has interacted with the COO^- of Xa. The complexation between the two oppositely charged polymers, Xa and Cs, did not affect the behaviour of the ester group, which showed, as expected, a less intense absorption around 1724 cm^{-1}. The FTIR spectrum of the Xa-Cs-Ne complex exhibits the characteristic absorption bands found for the raw products.

Surface morphology

The surface morphology of the Xa-Cs and Xa-Cs-Ne samples is illustrated in Fig. 2. Analysing the surfaces of the hydrogels, we can assert that Xa-Cs has a smooth surface with no apparent cracks and fractures. This result could be explained in terms of Xa and Cs chemical structure. The stability of the Xa-Cs complex is due to the electrostatic interaction between the carboxylic groups of Xa and the amino ionized groups of Cs. The three-dimensional network formed by two ionic bridges was described elsewhere in the case of the calcium-alginate gel.[25,26] As may be noted in the micrograph of the Xa-Cs-Ne complex, the drug is adsorbed on the Xa-Cs complex as distinct particles.

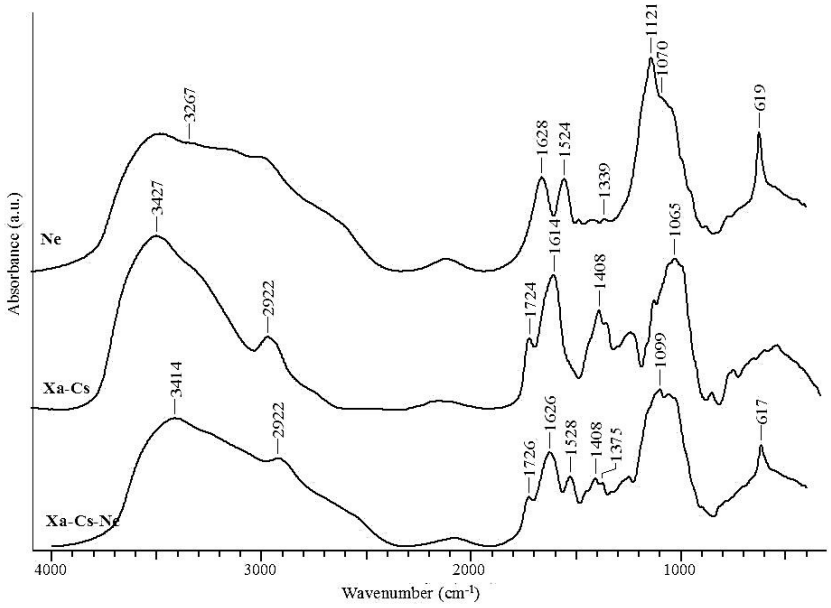

Figure 1: FTIRspectra of Ne, Xa-Cs and Xa-Cs-Ne

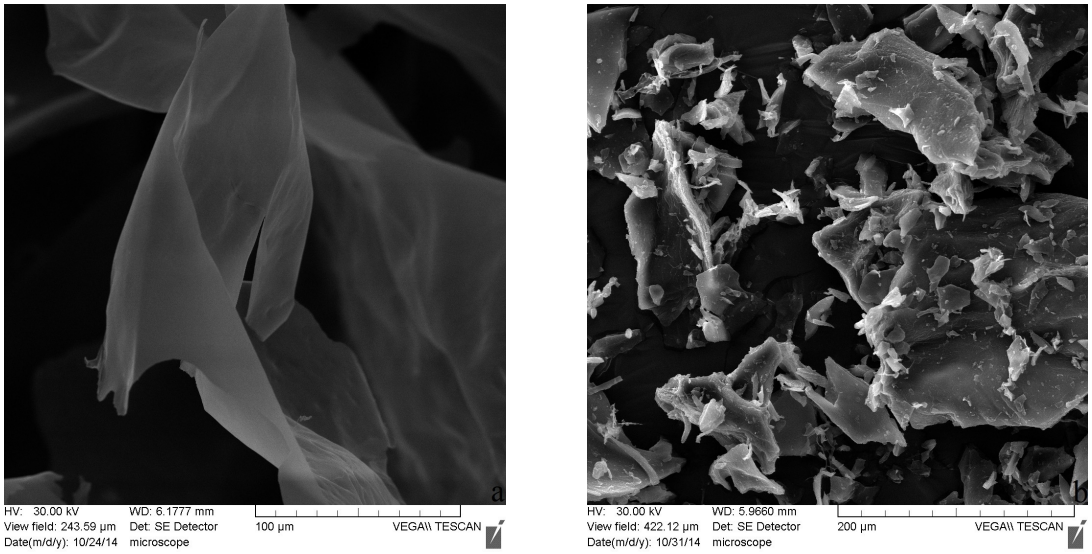

Figure 2: Scanning electron micrographs of (a) surface of Xa-Cs, (b) surface of Xa-Cs-Ne

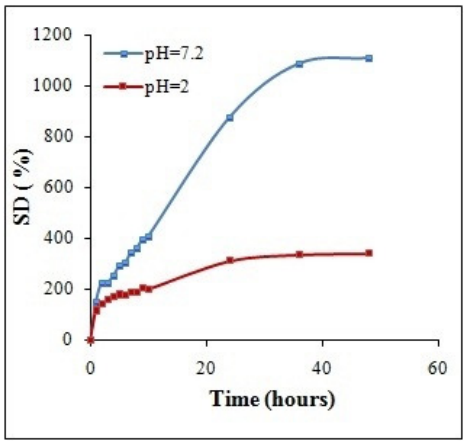

Figure 3: SD of Xa-Cs-Ne at (a) pH 2; (b) pH 7.2

Swelling behaviour of the hydrogel formulations

Fig. 3 shows the dynamic swelling behavior of the Xa-Cs-Ne tablets, in acidic and alkaline media. The ability of the hydrogel to swell in aqueous medium is influenced by the following parameters: the proportion between hydrophilic/hydrophobic links, the degree of cross-linking, the temperature, and especially the degree of ionization. All the samples achieve equilibrium swelling 48 hours after immersion. The complex reveals a pH-sensitive swelling behavior. SD was 220% at pH 2.0 and 1150% at pH 7.2, respectively. The carboxyl groups of Xa and the amino groups of Cs are involved in the Xa-Cs hydrogel formation. When the Xa-Cs hydrogel is loaded with Ne, additional bonds are formed between the carboxyl groups of Xa and the free amine groups of Ne. In basic medium, the cleavage of these bonds (pKa Ne 9.2) occurs, thus, large quantities of water can penetrate among the macromolecular chains, which leads to a much higher SD in basic medium compared to that in acidic environment. It has been previously shown that the Ne-Xa complex is stable at acidic pH and the drug is released only at basic pH.[27]

***In vivo* drug studies**

The rats were weighed by a digital electronic balance. There was a decrease in the weights of the rats treated with Ne when compared with the control group and complex group (Table 1). Exposure to the Ne-polymer complex did not affect the body weight gain of the treated animals.

Table 1
Values of gained body weight and statistical significance of differences among the groups

Group	I	II	III
Body weight gained (day 0-day 10)	48.33 ± 14.37	21.67 ± 12.1*	40.83 ± 13.19

Table 2
Values of biochemical parameters and statistical significance of differences among the groups

Group	I	II	III
Creatinine (mg/dl)	0.52 ± 0.03	0.60 ± 0.02	0.58 ± 0.02
Urea (mg/dl)	36.00 ± 1.10	59.30 ± 2.01*	41.80 ± 2.41**
Uric acid (mg/dl)	1.92 ± 0.29	2.86 ± 0.298	2.03 ± 0.29
Crp (mg/l)	0.013 ± 0.008	0.283 ± 0.040*	0.027 ± 0.010**

Values are mean ± SEM; *□<0.05 when compared to the normal control; **□<0.05 when compared to Ne induced non-treated disease control

Biochemical parameters

The biochemical parameters are presented in Table 2. It is well known that creatinine, urea and uric acid are markers of the renal function, and C-reactive protein (Crp) – of generalized injury and inflammation.[5,28,29] In our study, the serum concentrations of creatinine, urea and uric acid were elevated in group II and group III, compared to group I. An increase in these biochemical parameters was also reported in the literature in the case of Ne or other aminoglycosides, when the drug was administered in toxic doses.[30,31] However, the elevations in serum creatinine, blood urea and serum uric acid were attenuated by Xa-Cs coadministration.

The Crp level increased significantly in the case of raw Ne administration (group II). Values close to normal were achieved when a herbal formulation also comprising Ne was administered.[5] In the present study, the administration of the Xa-Cs-Ne complex normalized the serum Crp level, reducing drug toxicity, which was reflected in the improvement of all the biochemical parameters measured for group III. This effect is probably due the Cs from the Xa-Cs complex. Previous studies reported that Cs protected against kidney damage, in the case of gentamycin administration.[32]

Morphological study

Histological examination of kidney sections (Fig. 4) revealed normal aspects of the organs collected from the control group and kidney congestion, especially in cortical region, in the groups that receive raw Ne and the Xa-Cs-Ne complex. In another study, the histopathological examination of kidney sections revealed interstitial nephritis, in the case of Ne administration.[30]

According to the results (Fig. 5 a, b), the colon sections of the control group present a normal histological structure of the mucosa, submucosa, muscularis mucosae and serosa. The treatment with Ne (group II) was associated with disrupted architecture, injuries of the mucosal epithelium, aberrant crypts, an increased number of goblet cells, inflammatory cell infiltrate in the lamina propria and submucosa. Epithelial destruction associated with inflammation indicates that injuries were caused by the toxicity of Ne and not by the inflammatory elements involved. The results of the current study for group II are partially in agreement with those reported by Bose and co-workers.[5]They found that Ne changed the histology of the intestine without inducing inflammation. Previous histopathological examination of colon sections revealed changes in the crypt cells, blunting of villi, loss of microvilli and inflammatory cell infiltration of the lamina propria, in the case of Ne administration.[33] Histological analysis of colon sections from the Xa-Cs-Ne treated animals (Fig. 5 f,e) shows rare inflammatory infiltrate in the lamina propria and submucosa, as well as an increased number of goblet cells.

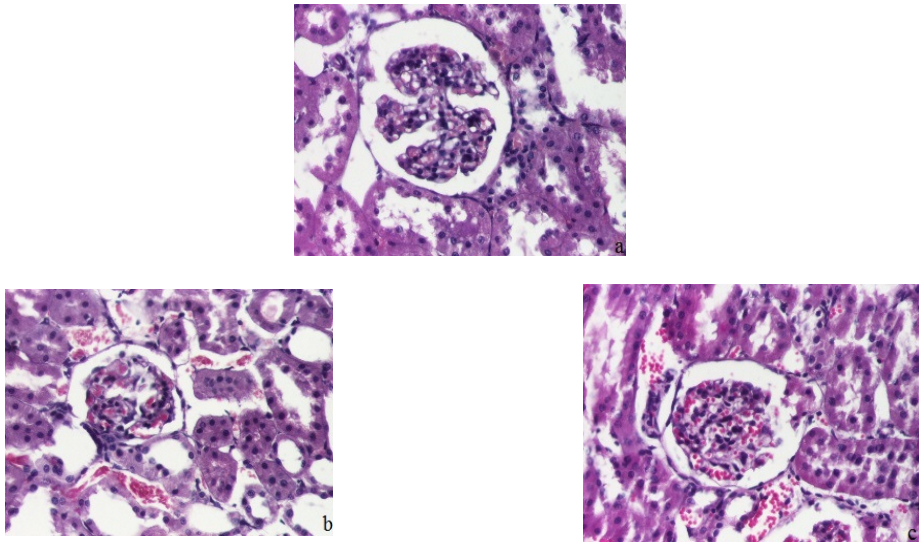

Figure 4: Representative microscopic images of hematoxylin-eosin-stained section of the kidney: (a) control rats, normal aspect (×200); (b) rats receiving raw Ne, congestion (×200); (c) rats receiving Xa-Cs-Ne complex, congestion (×200)

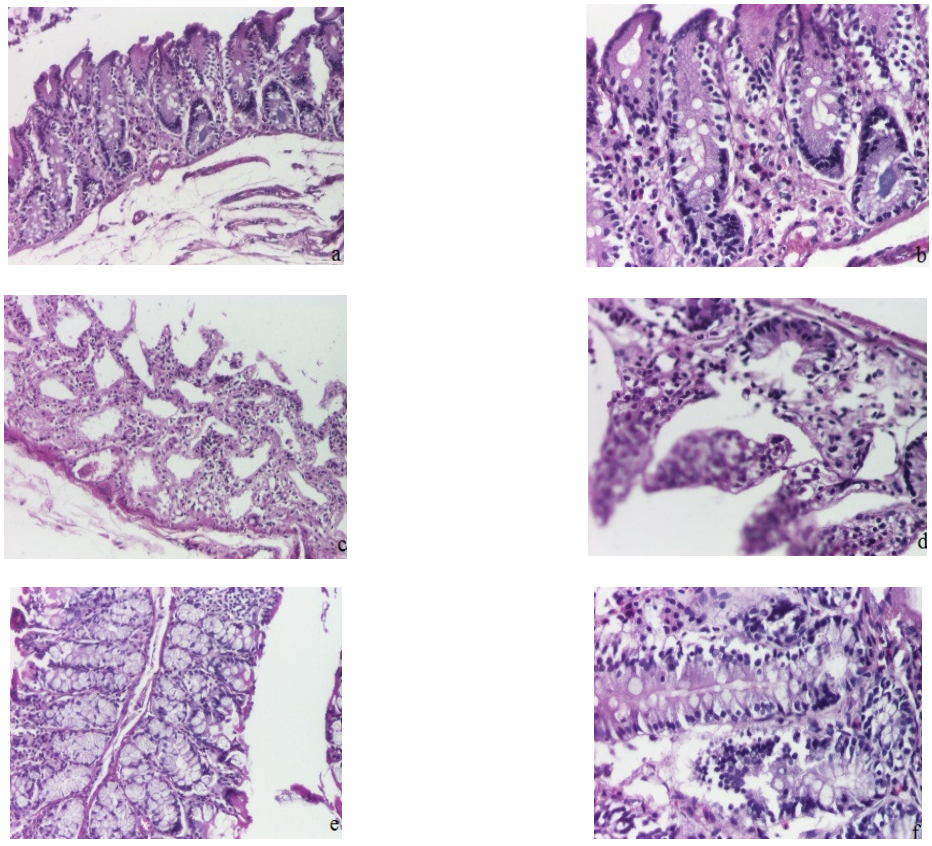

Figure 5: Representative microscopic images of hematoxylin-eosin-stained colonic tissue sections of rats from different experimental groups: control rats: (a) normal aspect (x100), (b) normal aspect (x200); rats receiving raw Ne: (c) aberrant small crypts (x100), (d) reduced crypts and epithelial injuries (x200); rats receiving Xa-Cs-Ne complex (e) increased number of goblet cells (x100), (f) increased number of goblet cells (x200)

The biochemical and histological findings referring to the Ne-polymer treated group are probably due to the result of matrix mucoadhesion, which enhances the drug absorption through the gastrointestinal mucosa without producing damage to the biological system.[15]

CONCLUSION

A Xa-Cs hydrogel was obtained as a result of complexation between the amino groups of Cs and the anionic groups of Xa. FTIR analysis confirmed ionic interactions between the Cs and Xa groups, as well as Xa-Cs-Ne hydrogen bonding interactions. The swelling behavior results demonstrated the pH-sensitive nature of this complex. Thus, the modification of creatinine, urea, uric acid and C-reactive protein levels in the serum and the morphological changes usually induced by Ne administration can be prevented by the coadministration of Xa-Cs and Ne.

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