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(54) Title: USE OF PHARMACEUTICAL COMPOUND CONTAINING CROTOXIN FOR THE TREATMENT OF MUSCLE DYSTONIAS

(57) Abstract: The present invention is related to the use of a pharmaceutical compound containing a toxin extracted from the rattlesnake (Crotalus durissus terrificus) venom, known as crotoxin, which can act as an agent provoking muscular paralysis. Medical uses of this invention formulation are described, which was developed for muscular pathological conditions whose muscular paralysis is desired, such as strabismus, blepharospams, and nystagmus, among others.

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"USE OF PHARMACEUTICAL COMPOUND CONTAINING CROTOXIN FOR THE TREATMENT OF MUSCLE DYSTONIAS"

The present invention is related to the use of a pharmaceutical compound containing a toxin extracted from the rattlesnake *{Crotalus durissus terrificus)* venom, known as crotoxin, which can act as an agent provoking muscular paralysis. Medical uses of this invention formulation are described, which was developed for muscular pathological conditions whose muscular paralysis is desired, such as strabismus, blepharospams, and nystagmus, among others.

The term strabismus first appears in HYPPOCRATES (460-377 B.C.) manuscripts. It comes from the Greek word *strabós,* meaning squinted or turned. The treatment for the various kinds of strabismus has been improving with time. The importance of preventing amblyopia and a better understanding of active and passive forces coordinating ocular movements are unquestionable.

The first reference in the literature on pharmacological treatment of strabismus was made in 1973 (SCOTT, A.B., ROSENBAUM, A., COLLINS, CC. Pharmacologic weakening of extraocular muscles. Invest Ophthalmol Vis Sci, v.12, p.924-927, 1973). These authors studied the effects of intramuscular injection of diisopropyl fluorophosphate (DFP), α-bungarotoxin (snake toxin), botulinum toxin type A and alcohol. They have observed that DFP and alcohol are very toxic and have no effect on the desired muscle paralysis. α-Bungarotoxin - the neurotoxin of a snake from India - has proved to be nontoxic in the applied concentrations and caused transitory paralysis in the extraocular muscle of monkeys. However, being of a short-duration, the transitory paralysis has not reached the proposed goal. The botulinum toxin type A also caused transitory paralysis in the extraocular muscle of monkeys, without any significant side effects. After in vivo experiment, they have concluded that botulinum toxin type A had proved to be appropriate for the human strabismus treatment. Following such discovery, other botulinum toxin therapeutic applications in ophthalmology were soon studied. The use of this drug in treating essential blepharospam is now the treatment of choice for this pathology (SCOTT, A.B., KENNEDY, R.A., STUBBS, H.A. Botulinum A Toxin Injection as a Treatment for Blepharospasm. Arch Ophthalmol, v.103, p.347-350, 1985).

Other toxins actuating in the neuromuscular junction could also be useful for strabismus therapy and for other diseases in which botulinum toxin type A has been applied successfully. The neurotoxin - crotoxin, due to its high toxicity - could also have a similar action of that botulinum toxin type A. Crotoxin is the main neurotoxin of

the rattlesnake *(Crotalus durissus terrificus)* venom. This the rattlesnake species is found in the Brazilian territory and is considered to be one of the most dangerous Western world rattlesnakes due to its venom high toxicity.

The rattlesnake *Crotalus durissus terrificus* venom does not usually generate a significant reaction on the spot of the bite. Its venom produces three main systemic changes: neurotoxic, myotoxic and coagulation changes. The neurotoxic activity is characterized by the blockage of neurotransmitter release through the peripheral neuron in the motor endplate in this way causing muscular paralysis. The clinical status is comprised of a discrete local manifestation and systemic intoxication that may become serious. The muscular paralysis caused by the rattlesnake *Crotalus durissus terrificus* venom may produce acute respiratory insufficiency that may lead to an individual's death. The specific treatment is to be accomplished with an anticrotalic serum or with a specific fraction of the antiophidian serum (CAMPOS, A.C.F.A. Efeito da crotoxina na fosforilaçao de proteinas da fraçao sinaptosomal de cortex de rato. Belo Horizonte, Instituto de Ciencias Biológicas. Departamento de bioquimica e imunologia da UFMG. Master's dissertation in biochemistry, 8Op.,2000).

Crotoxin provokes a three-stage blockage of acetylcholine release in the presynapse. In the first stage, a reduction of acetylcholine release is observed possibly related to the toxin link in the neuron membrane receptor. This stage lasts for approximately five minutes. Secondly, the neurotransmitter release is increased, which is related to the increased calcium concentration in the peripheral motor neuron cytoplasm. This stage progresses for 10 to 30 minutes. In the third stage, a progressive decrease occurs until a full blocking of acetylcholine is completed (HAWGOOD, B., BON, C. Snake venom presynaptic toxins. In: Handbook of natural toxins: reptile venoms and toxins. TU, AT. (ed). New York: Marcel Dekker Inc, p.5-32, 1990).

Crotoxin belongs to the b-neurotoxins family, which mainly acts at the presynaptic level. It is comprised of two subunits: a basic unit and an acidic one. Crotoxin's molecular weight is 23.5 KDa and that of the basic subunit is 14.3 Kda, with an isoelectric point of 8.9, and it is comprised of a single polypeptide chain with 123 aminoacids. The acidic subunit shows a molecular weight of 9.2 Kda, an isoelectric point of 3.8 and is constituted by three polypeptide chains (a, b, g) linked by disulphide bridges (AIRD, S.D., KAISER, 1.1., LEWIS, R.V., KRUGGER, W.G. Rattlesnake presynaptic neurotoxins: Primary structure and evolutionary origin of the acidic subunit. Biochemistry, v.24, p.7054-7058, 1985).

Both crotoxin subunits act in a coordinated and synergistic way. The basic portion performs a neurotoxic and phospholipasic activity. The acidic portion is pharmacologically inactive, although it increases the basic subunit toxicity. The basic portion link to the synaptic membranes is nonspecific, while the acidic portion is specifically linked and thus it potentiates the basic subunit action by reducing the nonspecific bond. Both portions are split after interacting with the synaptic membrane while the acidic portion is released (DÉLOT, E., BON, C. Model for the interaction of crotoxin, a phospholipase A2 neurotoxin, with presynaptic membranes. Biochemistry, v.32, p.10708-10713, 1993).

MUNIZ and DINIZ (1983) observed a decreased contraction of the smooth musculature in fragments of the longitudinal muscle of guinea pig myenteric plexus due to crotoxin action. The reduced contraction seems to be presynaptic as it remained unchanged when acetycholine was added (MUNIZ, Z.M., DINIZ, CR. Crotoxina afeta a resposta contratil do musculo liso induzida por estïmulo eletrico de campo. Arq Biol Tech, v.26, p.279-286, 1983). The release of acetycholine was significantly depressed by crotoxin, which validates the hypothesis of presynaptic blocking (ANADON, A., MARTINEZ-LARRANAGUA, M.R. Effects of crotoxin on automatic neuromuscular transmission in the guinea pig myenteric plexus and vas deferents. Toxicon, v.23, p.963-972, 1985).

Crotoxin also affects the kinetics of action potential as shown in frog skeletal muscle (ARAUJO, D.A.M., BEIRÃO, P.S.L. Effects of crotoxin on the action potential kinetics of frog skeletal muscle. Braz J Med Biol Res, v.26, p.1111-1121, 1993). Crotoxin increases the duration of action potential and diminishes the speed of polarization and repolarization. A resulting hypothesis is that crotoxin interferes with sodium channels thus retarding repolarization. The repose potential is not affected, which suggests inactivation of sodium channels and activation of potassium channels. Such changes in action potentials due to interference in ion channels do not depend on the crotoxin phospholipase activity and it is inhibited by substituting strontium for calcium (HAWGOOD, B.J., SMITH, J.W. The mode of action at the mouse neuromuscular junction of the phospholipase A2-crotapotin complex isolated from venom of the South American rattlesnake. Br J Pharm, v.61, p.607-614, 1977).

De LIMA and DINIZ (1985) observed that crotoxin inhibits the release of acetylcholine when stimulated by Tityustoxin, a toxin of *Tityus serrulatus* scorpion venom. This crotoxin action was decreased in the presence of bovine albumin, which is linked to the products by phospholipid hydrolisis. This reinforces the possibility of a

relevant crotoxin phospholipase action (De LIMA, M.E., DINIZ, CR. Crotoxin inhibits the release of acetylcholine induced by Tityus serrulatus scorpion venom. Toxicon, v.23, p.588, 1985).

Crotoxin may cause myotoxicity mainly due to phospholipase action, which induces degenerative necrosis of skeletal muscle irrespective of the action on the neuron (GOPALAKRISHNAKONE, P., DEMPSTER, D.W., HAWGOOD, B.J., ELDER, H.Y. Cellular and mitochondrial changes induced in the structure of murine skeletal muscle by crotoxin, a neurotoxic phospholipase A2 complex. Toxicon, v.22, p.85-98, 1984).

Neurotoxins by produced snake venoms differently affect the nervous system. They may be classified according to the mechanism of action and pharmacological properties and subdivided into two groups. The first group is comprised of postsynaptic action neurotoxins - called a-neurotoxins - producing nondepolarizing neuromuscular blocking. The second group is comprised of presynaptic action neurotoxins, classified as b-neurotoxins. The latter usually have more complex molecules than those of a-neurotoxins and show phospholipase action (LEE, CY. Chemistry and pharmacology of polypeptide toxins in snake venoms. Annu Rev Pharmacol, v.12, p.256-286, 1972). Some a-neurotoxin examples are: a-bungarotoxin of Taiwan snake, *Bungarus multicinctus* and the a-toxin of *Naja nigricollis.* Examples of b-neurotoxins are as follows: crotoxin of South American rattlesnake, *Crotalus durissus terrificus,* b-bungarotoxin of S. *multicinctus* snake and notoxin Australian tiger snake, *Notechis scutatus scutatus.*

Crotoxin is ranked as a b-neurotoxin. Such toxins block neuromuscular transmissionem at the presynaptic level and have phospholipase activity and they may be monomeric or polymeric toxins. Crotoxin belongs to the polymeric neurotoxin group as it shows to have two chains, a basic chain and an acidic chain (HORST, J., HENDON, R.A., FRAENKEL-CONRAT, H. The ative components of crotoxin. Biochem Biophy Res Commun, v.46, p.1042-1 047, 1972).

Observations relating to irreversible and reversible ihnibition of A2 phospholipase activity of b-neurotoxins suggest that their enzymatic activity is a necessary condition for their neurotoxic action. Many evidence instances show that b-neurotoxins do not recognize the same molecular target (CHANG and SU, 1980; REHM and BETZ, 1982). Such neurotoxins of snake venoms block neurotransmission at the neuromuscular junction, primarily at the presynaptic level, by interfering with acetylcholine release, causing death by respiratory stoppage. In addition to

neurotoxicity, different PLA2 of snake venoms present other types of toxicity, including myotoxicity, anticoagulant e and proinflammatory effects (KINI e EVANS, 1989; VADAS et al., 1981).

Crotoxin has been classified as a postsynaptic neurotoxin, although its presynaptic effect seems to be its most important pharmacological action as it blocks neurotransmitter release (HAWGOOD, B., BON, C. Snake venom presynaptic toxins. In: Handbook of natural toxins: reptile venoms and toxins. TU, A.T. (ed). New York: Marcel Dekker Inc, p.5-32, 1990).

Based on data indicating that crotoxin acts as a neuromuscular blocking, the present patent researchers have decided to verify whether it can be used for inducing extraocular muscle paralysis in rabbits. In addition to analyzing paralysis induction, The permanence period of the effect on musculature and the possible side effects have also been verified.

The patent US20060045875A1 describes a pharmaceutical compound containing crotoxin for treating and preventing retroviral infections in cells of mammals. This invention includes identification of beta-neurotoxins of snakes of the genus *Crotalus* able to prevent infections caused by the HIV virus and its replication in cells. The selected retroviruses include those of the genus Lentivirus (HIV-1, HIV-2, SIV, EIAV, BIV and FIV).

The patent US2004000917143 discloses a pharmaceutical compound presenting one of the following toxins: crotoxin, mojavetoxin and drug carriers for treating chronic pain, mainly painful cases associated to cancer. Crotoxin is believed to be preferably taken from snake of the genus *Crotalus durissus terrificus* and mojavetoxin from snakes of the genus *Crotalus scutulatus scutulatus.* Additionally, the present invention describes an effective amount of acetylsalicylic acid added to this compound so as to make it - together with the toxin - possess a synergistic effect in promoting analgesia.

The patent EP0246861A2 describes the chromatographic insulation of A and B of crotoxin taken from the genus *Crotalus durissus terrificus* for treating carcinoma and infectious and endocrine vascular disorders as well as for the treatment of pains.

The patent US1 990000460508 describes a pharmaceutical compound based on crotoxin, with cytotoxic activity coming from the A2 insulated phospholipase of the snake of the genus *Crotalus durissus terrificus* for treating several kinds of malign tumors in advanced stages. A method for purifying active components, the preparation

of a pharmaceutically acceptable formulation and a therapeutic method for malign tumors are also described.

The patent US1 981 000251745 gives an account of stable compounds of venoms and/or fractions of snake venoms with useful pharmacological activity for the treatment of neurological disorders, particularly those caused by immune system malfunction. The present invention presents compounds that include a postsynaptic component able to link itself to the acetylcholine receptor, which is a presynaptic component able to inhibit the acetylcholine release and a viperine component able to stimulate the immune system. Methods for preparing the formulation are also described.

The patent BR2004000001702 describes peptide compounds derived from snakes, e.g., *Crotalus durissus terrificus,* for analgesic use that are useful for treating, preventing and diagnosing painful conditions mediated by opium receptors. Pharmaceutical compounds and methods for preparing and purifying analgesic compounds including their use and identification are also described.

Defining the dosage of crotoxin to be used in the present patent experiment was based on the DL-50 of this toxin, as compared to the DL-50 of botulinum toxin type A (SCOTT, A.B., ROSENBAUM, A., COLLINS, CC. Pharmacologic weakening of extraocular muscles. Invest Ophthalmol Vis Sci, v.12, p.924-927, 1973). As there was no bibliographic reference on the crotoxin effects on the extraocular musculature, a 10,000 times variation of crotoxin concentration was used for the analysis of its effects on paralysis induction of extraocular musculature and its possible side effects. The botulinum toxin type A (Botox®) was used for comparison with crotoxin effects.

The tolerance level of toxins was good and the rabbits did not show systemic prostration signs or changes in feeding habits. Except for side effects, all rabbits did not show signs of any disease during experimentation.

The assessment of paralysis or paresis of superior straight muscles that were submitted to crotoxin and botulinum toxin type A application was made through electromyography in the experimentation. The ocular extrinsic musculature of the rabbit is of the indented type. This kind of musculature is functionally comprised of motor units in which the individual motor cell axons innervate many muscular fibers. Motor units are the smallest functional units of the musculoskeletal system. Information on the function of such systems is particularly obtained by means of electromyography. Several action potentials of many motor end-plates put together compose the muscle action potential. This action potential starts at the motor end-plates and is triggered by

a nervous impulse efferent from the neuromuscular junction. Then, it spreads throughout the muscular fibers stimulating contraction. Intensity of contraction partly depends on the number of motor end-plates that are being activated (recruitment) and partly on the frequency with which neurons send their impulses to muscular fibers. As for peripheral nerve lesions, electromyography can be used in a much more precocious stage than any other method of determining paralysis occurrence and of verifying whether regeneration is satisfactorily in progress.

In case the peripheral motor neurons supplying a muscle are not completely destroyed the following paralysis will be partial (or paretic), proportional to the number of affected cells. An electromyograph is able to reveal light action potentials in deinnervated muscles, i.e. those which have lost their innervation. Such potentials are called fibrillation potentials that seem to be stemmed from isolated muscle fibers, which have lost their innervation. The source of fibrillation is not yet completely determined, but it has been stated that it is due to some *sensitivity* of the terminal plate muscular portion after deinnervation. Absence of a normal transmitter - acetylcholine - possibly makes muscle fiber to respond to the small amount of acetylcholine present in the blood stream.

When experiments were conducted for the present patent, electromyographic registers were performed by introducing an electrode into the superior straight muscle of a rabbit under local anesthesia. A first examination was accomplished in all animals two days after applying the toxin. Five electromyographic examinations were performed in rabbits numbered 1 to 8 and four examinations were accomplished in rabbits 9 to 12. Rabbits were separated into groups of two and the two rabbits of each group presented corresponding electromyographic alterations in most registers, which made separate description unnecessary.

Rabbits of group 1, who received a 0.015 mg crotoxin application, presented discreet electromyographic alterations. Fibrillation was not observed in this group and action potentials were kept practically normal immediately after the crotoxin application. Irritability signs were observed in the neuromuscular sheaths after two weeks, although they were discreet and have disappeared by the time of the following examination.

Rabbits of group 2, numbered 3 and 4, received 0.15 mg of crotoxin and showed their action potentials within normal limits, whose amplitude, however, was smaller than that of group 1. These initial alterations were not verified in the following examinations. The signs of irritability in this group were tardy and short-lived and there was neither recruitment reduction nor reinnervation.

Rabbits in group 3 received 1.5 mg of crotoxin and their electromyographic alterations were more significant. Potentials shown in the first examination were rare and of decreased amplitude. These potentials showed signs of irritability nonspecifics of neuromuscular. In the second examination, potentials were better, but some motor units were still responding (the number of units recruited for the effort remained reduced). Sixteen days past, potentials practically returned to normality.

Group 4 - to which botulinun toxin type A was applied - immediately presented blocked capacity of capturing potentials together with irritability signs (fibrillations). By the time of the second examination nine days after, fibrillation signs associated to rare normal and low-amplitude potentials still remained. The recovery was gradual after a period of approximately sixteen days with visible signs of regeneration (polyphasics), which show an asynchronous recruitment (recent regeneration). After one month, irritability signs (fibrillations) disappeared and motor units presented normal amplitude and duration when contracting. The interference pattern still proved to be incomplete but no polyphasics (asynchrony) were not found, which may suggest that regeneration was almost complete. During the last examination, almost two months later, electromyographic records were practically normalized. SCOTT, ROSENBAUM and COLLINS (1973) obtained normal electromyographic records after three months and a half with an animal presenting strabismus for eight months secondary to the application of botulinum toxin type A (SCOTT, A.B., ROSENBAUM, A., COLLINS, CC. Pharmacologic weakening of extraocular muscles. Invest Ophthalmol Vis Sci, v.12, p.924-927, 1973).

Rabbits 9 and 10 of group 5, who received 15 mg of crotoxin, showed an immediate reduced amplitude of potentials and reduced number of functioning motor units, a process similar to myolisis. Signs of asynchronic recruitment of motor units (polyphasics) were identified after two weeks; nonspecific signs of irritability (fibrillations) became evident from regeneration up to normality.

I group 6, rabbit 11 received 150 mg of crotoxin at a volume of 100 ml and rabbit 12 received a solution of 75 mg at a 50 ml volume. Such a difference could explain the more visible local alterations in rabbit 11, including leukoma. As to electromyographic records, rabbit 11 also presented more significant changes; an immediate and complete contraction blocking, which gave a false impression of muscle absence. During the second week, rabbit 11 remained totally without potentials and signs of nonspecific irritability at rest. In rabbit 12, irritability signs were less significant and rare, despite the presence o functioning units (action potentials). One month past,

the action potentials of rabbits 11 and 12 remained normal during contraction with a reduced number of motor units however, mainly as for rabbit 11. The last examination showed that the animals presented nonspecific signs of membrane irritability.

The electromyographic findings showed that crotoxin provokes a reversible paralysis of the muscular activity, which was proportional to the applied toxin dose in accordance with the findings by ARAUJO and BEIRÃO (1993) (ARAUJO, D.A.M., BEIRAo, P.S.L. Effects of crotoxin on the action potential kinetics of frog skeletal muscle. Braz J Med Biol Res, v.26, p.1 111-1121, 1993). Reduced action potentials were observed, mainly as for crotoxin concentrations above 1.5 mg. The recovery from paralysis was the gradual and electromyographic records were practically normalized after a two-month follow-up. The applied botulinum toxin type A (two units of Botox®) also caused a reversible paralysis. The muscle action potentials that received botulinum toxin type A started to recover after nine days and they were almost normalized two months past. This shows that induced paralysis caused by certain crotoxin concentrations lasts for a period similar to that of botulinum toxin type A (Botox®).

Morphological changes observed in mice through electronic microscopy showed that high doses of crotoxin affect nervous terminals, skeletal muscle and axons. Such effects were also verified only after applying the PLA2 crotoxin subunit, and this did not happened with the other subunit. The first structures to be affected are: sarcolemma, mitochondrion and sarcoplasmic reticulum (HOWARD, B.D. Presynaptic polypeptide neurotoxins. Trends Pharmac Sci, v.3, p.167, 1982). In 1984, GOPALAKRISHNAKONE et al., found that the beginning of myonecrosis caused by crotoxin was provoked by a gradual loss sarcolemmal integrity due to the hydrolysis of the phospholipic component. The muscle fibers regeneration was swiftly completed (GOPALAKRISHNAKONE, P., DEMPSTER, D.W., HAWGOOD, B.J., ELDER, H.Y. Cellular and mitochondrial changes induced in the structure of murine skeletal muscle by crotoxin, a neurotoxic phospholipase A2 complex. Toxicon, v.22, p.85-98, 1984).

One animal of each group was sacrificed in our experiment for an anatomo-pathological study. The histological changes found were proportional to the crotoxin concentration and localized only on the application region. Necrosis of muscle fibers was not found in none of the analyzed samples. A discreet endomysial fibrosis only was observed in the smallest concentrations (0.015 mg and 0.15 mg) as well as mononuclear infiltrate in some regions. The inflammatory process was also discreet in the 1.5mg crotoxin concentration. However, signs of myophagocytosis, vacuolate fibers

(a degenerative sign) and central-nucleus fibers (a sign of regeneration) could be found. These changes were similar to those found in the rabbit who received Botox®.

Some studies have shown that botulinum toxin type A causes alterations in the musculature to which it has been applied. Such disturbances are proportional to the toxin dosage (BORODIC, G.E., FERRANTLE, R., PEARCE, L.B., SMITH, K. Histologic assessment of dose related diffusion and muscle fiber response after therapeutic botulinum A toxin injections. Mov Disord, v.9, p.31-39, 1994). Similarly to the crotoxin studies, the histologic changes caused by botulinum A toxin have shown to be reversible (PORTER, J.D., STREBECK, S., CAPRA, N.F. Botulinum induced changes in monkey eyelid muscle. Arch Ophthalmol, v.109, p.396-404, 1991). The changes observed in groups 5 and 6, which received 15 mg and 150 mg crotoxin concentrations, respectively, were more significant than those found in previous groups and they were more notable in the group with the greater concentration. Signs of degenerated fibers (vacuolate fibers), loss of grooves and endomysial fibrosis were more evident. Muscle fiber signs of regeneration (characterized by the central nucleus) were also observed.

The muscular paralysis induced by crotoxin could be verified by means of eletromyographic records. It has been observed that crotoxin in certain concentrations had an effect similar to that of the already known botulinum toxin, which provoked only discreet local and histological changes.

Based on such crotoxin use observations, it is believed that the crotoxin pharmacological action may be useful in pathological conditions to which botulinum toxin has been successfully applied. The following examples describe the present invention with more details, but in an unlimited manner.

EXAMPLE 1: PREPARING CROTOXIN COMPOUND

CROTOXIN

The crotoxin used for this study was offered by the *Divisao de Imunobiológicos da Fundagao Ezequiel Dias* - FUNED (Immuno-biological Division of the Ezequiel Dias Foundation - Belo Horizonte, Minas Gerais) that has taken the rattlesnake *(Crotalus durissus terrificus)* venom, purified it and performed the DL-50 study.

CROTOXIN PURIFICATION

Crotoxin in its pure state was obtained from the South American rattlesnake *{Crotalus durissus terrificus)* venom. The snake was put to sleep in a dry-ice mist of CO_2. The animal's glands were pressed for collecting the venom in an appropriate recipient.

CROTOXIN ISOELETRIC PRECIPITATION.

The product was collected and lyophilized at the temperature of 40° C. The dehydrated venom was dissolved in 0,02 M phormiate buffer, pH 3,5 in 0,2 M sodium chloride. Then, the solution was centrifuged at 10,000 rpm for 15 minutes, and the clear coagulated protein floating fractions being separated. The limpid solution was applied to the Sephadex G-100 column equilibrated in the same phormiate buffer.

CHROMATOGRAPHY ON SEPHADEX G-100 GEL

The chromatography presented the highest peak of protein concentration corresponding to crotoxin. The peak was separated and lyophilized. A rechromatography in the same column and the same conditions was made so as to guarantee a high purity crotoxin grade. In this last stage, the chromatographic profile presented a single symmetric peak by superposing protein curves (given by absorbance at 280 nm), which characterized a high purity. This purity was later verified by SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis. The lyophilized crotoxin was dissolved in saline solution and its toxicity was later assessed by LD-50in mice.

DETERMINATION OF LD-50

This solution toxicity was determined in mice by intraperitoneal LD-50 ranging from 18 to 22 grams. Female and male mice were used in groups comprised of 8 animals by dose, the reading of which was made after 48 hours. The estimated LD-50 for a person weighing 70 kg would correspond to 3,500 LD-50 in a mouse.

Botox® is the most frequently used botulinum toxin, a unit of which corresponds to 1 LD-50. In treating strabismus 5 to 10 units are usually applied in each extraocular muscle. As for blepharospasm, 25 to 50 units are used and 100 units have been used in great muscles (e.g., those of inferior members).

EXAMPLE 2: EXPERIMENTATION IN ANIMALS

All procedures were accomplished in accordance with the Brazilian Law 6,638, as of May 1979. This legal statute has fixed rules for educational and scientific use of vivisection of animals. According to this law, researches must always be accomplished in such a way as to not to cause animal suffering.

Twelve New Zealand albino rabbits with an average weight of 2.5 kg were used for this study. The rabbits were divided into six groups with 2 specimens in each group. The initial crotoxin dose used was based on a comparison between the minimum lethal dose (LD-50) of crotoxin and that of botulinum toxin type A (SCOTT, ROSENBAUM

and COLLINS, 1973) applied in mice. The botulinum toxin type A used in this study had an LD-50 of $1x10^{-4}$ mg and the dose applied to cause extraocular muscle paresis in *rhesus* monkeys was of $1x10^{-5}$ to $1.6x10^{-3}$ mg (0.01 a 1.6 ng). The volume applied ranged from 5 to 500 ml. A partial paralysis of extraocular straight muscles was obtained, which lasted from 2 weeks to 8 months. A traded unit of botulinum toxin type A (Botox®) is equal to LD-50 in mice.

The crotoxin used had an LD-50 of 1.5 mg. Toxicity is determined by the minimum lethal dose that kills 50% of inoculated animals (LD-50), by using conventional-bred Swiss mice weighing 18-22g. The estimated efficacious minimum dose of crotoxin - as compared to that of botulinum toxin type A of the study by SCOTT, ROSENBAUM and COLLINS as of 1973 - would be 0.15 mg.

Based on these estimates, three crotoxin concentrations were chosen. The dosage for the first group was ten times smaller than the minimum theoretically efficacious dose; the second group received the estimated those; the third group, a dose tern times greater than the estimated one, which was equal to LD-50 of crotoxin.

Rabbits of group 4 received 2 units of botulinum toxin type A. As the crotoxin side effects were small in the first inoculated rabbits, two other groups were formed whose crotoxin concentrations applied were 15 mg and 150 mg, respectively.

Crotoxin and botulinum toxin type A were injected into the right superior straight muscle of each rabbit with an insulin syringe (Unijet, Plascalp Produtos Cirúrgicos Ltda., Feira de Santana, BA). The application of toxins was not accomplished by means of electromyographer as the ideal spot for toxin injection had been previously assessed through electromyography. The application was performed after instillation of an anesthetic coilyrium of proxymetacaine chloride at 0.5% (Anestalcon® - Alcon Laboratories do Brasil, Sao Paulo, SP) and under direct visualization of the superior rectus muscle. The needle was transconjunctival^ introduced into the muscle and the injection was performed at approximately 4mm after the scleral insertion of the superior rectus muscle. The applied volume was of 100 ml, except for rabbit n^0 12, who received 50 ml. The solution applied to rabbit 12 contained a crotoxin concentration of 150 mg in 100 ml: this means that this rabbit received half a dose as compared to that for rabbit n^0 11. The dosage applied to each rabbit is described as follows:

Group 1	=>	rabbits 1 and 2 =>	0.015 µg of crotoxin
Group 2	=>	rabbits 3 and 4 =>	0.15 µg of crotoxin

Group 3	=>	rabbits 5 and 6 =>	1.5 µg of crotoxin
Group 4	=>	rabbits 7 and 8 =>	two units of botulinum toxin type A (Botox®)
Group 5	=>	rabbits 9 and 10 =>	15 µg de crotoxina
Group 6	=>	rabbit 11 =>	150 µg of crotoxin
		and rabbit 12 =>	75 mg of crotoxin

The following table shows procedures used in the 12 rabbits:

RABBIT	TOXIN APPLIED	VOLUME APPLIED	DATE OF APPLICATION	MUSCLE APPLIED
1	Crotoxin 0.015 µg	100 µl	08/10/2000	SR RE*
2	Crotoxin 0.015µg	100 µl	08/10/2000	SR RE
3	Crotoxin 0.15 µg	100 µl	08/10/2000	SR RE
4	Crotoxin 0.15 µg	100 µl	08/10/2000	SR RE
5	Crotoxin 1.5 µg	100 µl	08/10/2000	SR RE
6	Crotoxin 1.5 µg	100 µl	10/08/2000	SR RE
7	Botox® 2 units	100 µl	08/10/2000	SR RE
8	Botox® 2 units	100 µl	08/10/2000	SR RE
9	Crotoxin 15 µg	100 µl	08/17/2000	SR RE
10	Crotoxin 15 µg	100 µl	08/17/2000	SR RE
11	Crotoxin 150 µg	100 µl	08/17/2000	SR RE
12	Crotoxin 75 µg	50 µl	08/17/2000	SR RE

** SRRE = Superior rectus of right eye*

Local and systemic side effects on the rabbits were daily observed during the first week. An analysis of possible systemic side effects was accomplished by observing the animals' behavior. Laboratory exams for assessing hepatic functions were not performed. Later on, such an examination was made twice a week up to the experiment conclusion. Electromyographic examinations were performed in order to assess toxin actions. One animal of each group was sacrificed after the last electromyographic examination for anatomo-pathological study.

The experiment was well tolerated by the rabbits and no behavioral change was observed after the application of crotoxin and the botulinum toxin type A (Botox®). The animals did not show signs of prostration or loss of appetite after both toxin assays. A day after the toxin application, rabbits 1, 2, 3, 5, 6, 7, 8, 9, 10, 11 and 12 showed a

certain degree of ptosis, which was very discreet and lasted for only two days in rabbits 1, 2, 3, 5 and 6.

Seven days past after the application, rabbits 7, 8, 11 and12 still showed to have ptosis. Rabbit 8 presented the longest period of palpebral ptosis: fourteen days.

There was discreet conjunctival hyperemia on the application spot in rabbits 1, 2, 3, 4, 7 and 8, which did not surpassed 48 hours. The conjunctival reaction was moderate in rabbits 5 and 6, gradually receding in seven days. An accentuated purulent conjunctival hyperemia was observed in rabbits 9, 10, 11 and 12. These rabbits still maintained moderate signs of this reaction seven days after and they showed to have only a discreet reaction fourteen days after the toxin application.

The most significant reaction was shown by rabbit 11. In addition to the conjunctival reaction, it showed a corneal edema that completely disappeared in fourteen days. After this performance, this rabbit showed a definitive leukoma in the affected cornea.

EXAMPLE 3: ELECTROMYOGRAPHIC EXAMINATION

The electromyographic examinations were performed at the Instituto de Neurofisiologia Clinica de Minas Gerais (Clinical Neurophisiology Institute of Minas Gerais), in Belo Horizonte. The animals received an instillation of 3 drops of proxymetacaine chloride collyrium at 0.5% (Anestalcon® - Alcon Laboratórios do Brasil, Sao Paulo, SP, Brazil) before the examination.

The electromyographer used was a Neuropack Four Mini Evoked Potential Measuring System MEB-5304K (NIHON KOHDEN, Tokio, Japan). A bipolar concentric electrode with central platinum wire stainless steel cannula was used. The electrode was 37 mm long, with a needle diameter of 0.46 mm with a record area of 0.07 mm (Teca Corporation, catalog n^0 53156, New York, USA). A Velcro® strap (Teca Corporation, catalog n^0 GE-V, New York, USA), soaked in saline solution, was placed around the rabbits' ears for grounding the system.

The electromyographic examinations of rabbits 1 to 8, which were performed two days after the toxin applications, are recorded in table 2:

Superior straight Muscle of rabbit n°	Myoelectric activity		
	Repose	Moderate Contraction Maximum Action potential	Contraction

	Increased Insertion activity	Fibrillation	Positive waves	Bizarre discharge	Fasciculation	Repose silence	P.A.U.M.N *	Low amplitude potential	Giant potentials	Long polyphasics	Short polyphasics	Absence of potentials	Normal interference pattern	Incomplete interference pattern	Motor unit interference pattern
1 Crtx 0.015µg								X							
2 Crtx 0.015 µg								X							
3 Crtx 0.15 µg							X								
4 Crtx 0.15 µg							X								
5 Crtx 1.5 µg							X								
6 Crtx 1.5 µg							X								
7 Botox ® 2 unidades		X										X			
8 Botox ® 2 unidades		X		X								X			

* *Normal action potentials of motor units.*

- *Group 1 - Rabbits 1 and 2 - Crotoxin at 0.015 mg:* There were no signs of irritability of neuromuscular sheaths. The observed potentials at contraction showed normal amplitude and duration results, without major changes in fiber recruitment, which was only incomplete.
- *Group 2 - Rabbits 3 and 4 - Crotoxin at 0.15 mg:* The same pattern observed for dosage 0.015 mg was obtained for this group. Despite the fact that potentials were within normality limits, the amplitudes verified were smaller than the lower-dosage potentials.
- *Group 3 - Rabbits 5 and 6 - Crotoxina 1.5 mg:* In spite of absent membrane irritability signs, potentials obtained for this group were rare and of reduced. The findings suggest a conduction block between fibers and/or loss of muscular fibers.
- *Group 4 - Rabbits 7 and 8 - Botulinum Toxin Type A (2 U):* Signs of neuromuscular sheath irritability at repose were observed. There were no fiber response - in this case, by conduction block at the neuromuscular junction. There was no fiber response, i.e., no motor unit to contraction was observed. The presence of fibrillation potentials may suggest functional deinnervation.

Table 3 presents the findings of electromyographic examinations accomplished nine days after toxin application in rabbits n^0 1 to 8, and two days past in rabbits n^0 9 to 18:

Superior straight Muscle of rabbit nº	Myoelectric activity		
	Repose	Moderate Contraction Maximum Action potential	Contraction

	Increased Insertion activity	Fibrillation	Positive waves	Bizarre discharge	Fasciculation	Repose silence	P.A.U.M.N *	Low amplitude potential	Giant potentials	Long polyphasics	Short polyphasics	Absence of potentials	Normal interference pattern	Incomplete interference pattern	Motor unit interference pattern
1 Crtx 0.015µg	X						X								
2 Crtx 0.015 µg							X								
3 Crtx 0.15 µg							X								
4 Crtx 0.15 µg							X								
5 Crtx 1.5 µg							X								
6 Crtx 1.5 µg							X								
7 Botox ® 2 unidades		X					X								
8 Botox ® 2 unidades		X					X								
9 Crtx 15 µg								X							
10 Crtx 15 µg								X							
11 Crtx 150 µg												X			
12 Crtx 75 µg												X			

* *Normal action potentials of motor units.*

- *Group 1 - Rabbits 1 and 2 - Crotoxin at 0.015 mg:* The action potentials captured were normal and with no irritability signs (instability of membranes).
- *Group 2 - Rabbits 3 and 4 - Crotoxin at 0.15 mg:* activity similar to that of groupi .
- *Group 3 - Rabbits 5 and 6 - Crotoxin at 1.5 mg:* there were no signs of irritability, but the capture of potentials (normal ones) showed few motor units responding, just like what happened in group 4.
- *Group 4 - Rabbits 7 and 8 - Botulinum Toxin Type A (2U):* signs of membrane irritability persist, but now with rare normal potentials captured during contraction.
- *Group 5 - Rabbits 9 and 10 - Crotoxin at 15 mg:* without signs of membrane irritability, although the rare captured potentials wereof low amplitude or blocked as to response to stimuli.
- *Group 6 - Rabbits 11 and 12 - Crotoxin at 150 mg and 75 mg:* the electromyographic record was completely silent as if there was no muscle.

Table 4 shows the findings of electromyographic examinations accomplished sixteen days after toxin applications in rabbits 1 to 8, and nine days past in rabbits 9 to12:

Superior straight Muscle of rabbit nº	Myoelectric activity														
	Repose						Moderate Contraction Maximum Action potential						Contraction		
	Increased Insertion activity	Fibrillation	Positive waves	Bizarre discharge	Fasciculation	Repose silence	P.A.U.M.N *	Low amplitude potential	Giant potentials	Long polyphasics	Short polyphasics	Absence of potentials	Normal interference pattern	Incomplete interference pattern	Motor unit interference pattern
1 Crtx 0.015µg				X			X								
2 Crtx 0.015 µg							X								

3 Crtx 0.15 µg	X						X								
4 Crtx 0.15 µg							X								
5 Crtx 1.5 µg	X						X								
6 Crtx 1.5 µg	X						X								
7 Botox ® 2 unidades		X	X				X			X					
8 Botox ® 2 unidades		X	X				X								
9 Crtx 15 µg	X						X			X					
10 Crtx 15 µg	X						X			X					
11 Crtx 150 µg												X			
12 Crtx 75 µg							X								X

• *Normal action potentials of motor units.*

- *Group 1 - Rabbits 1 and 2 - Crotoxin at 0.015 mg:* rabbit n^0 1 showed signs of intense and nonspecific irritability with high frequency discharges. There were no such signs in rabbit n^0 2.
- *Group 2 - Rabbits 3 and 4 - Crotoxin at 0.15 mg:* no difference as compared to rabbit n^0 2.
- *Group 3 - Rabbits 5 and 6 - Crotoxin at 1.5 mg:* normal potentials during contraction with more intense irritabiity signs in comparison with those for groups 1 and 2.
- *Group 4 - Rabbits 7 and 8 - Botulinum Toxin Type A (2U):* the signs of neuromuscular irritability were more intense with normal amplitude potentials and duration of contraction, associated to long polyphasic potentials, which characterized asynchronous recruitment, typical of recent reinnervation. In spite of recruitment pattern not being normal yet, the response was not rarefied.
- *Group 5 - Rabbits 9 and 10 - Crotoxin at 15 mg:* signs of nonspecific irritability were also present, but asynchronous recruitment signs of motor units (polyphasics) during contraction were verified.
- *Group 6 - Rabbits 11 and 12 - 150 mg e 75 mg:* rabbit 11 received a 100 ml volume, whose predominant characteristic was thorough absence of potentials with nonspecific irritability signs at repose. Rabbit 12 received a 50 ml volume, and showed smaller irritability and, despite the existence of functioning units, they were rare.

Table 5 shows the findings of electromyographic examinations accomplished twenty-three days after toxin applications in rabbits 1 to 8, and sixteen days past in rabbits 9 to12:

Superior straight Muscle of rabbit n°	Myoelectric activity														
	Repose						Moderate Contraction Maximum Action potential						Contraction		
	Increased Insertion activity	Fibrillation	Positive waves	Bizarre discharge	Fasciculation	Repose silence	P.A.U.M.N *	*Low amplitude potential*	Giant potentials	Long polyphasics	Short polyphasics	Absence of potentials	Normal interference pattern	Incomplete interference pattern	Motor unit interference pattern
1 Crtx 0.015µg								X	X			X			
2 Crtx 0.015 µg						X		X							X
3 Crtx 0.15 µg				X											
4 Crtx 0.15 µg						X		X	X		X				
5 Crtx 1.5 µg				X				X							

6 Crtx 1.5 µg				X			X	X						
7 Botox ® 2 unidades						X	X		X				X	
8 Botox ® 2 unidades						X	X						X	
9 Crtx 15 µg						X	X		X			X		
10 Crtx 15 µg						X	X		X			X		
11 Crtx 150 µg						X	X							X
12 Crtx 75 µg						X	X						X	

** Normal action potentials of motor units.*

- *Group 1 - Rabbits 1 and 2 - Crotoxin at 0.015 mg:* normal potentials and recruitment.
- *Group 2 - Rabbits 3 and 4 - Crotoxin at 0.15 mg:* there are no signs of irritability and the units were normal during contraction, but there were signs of asynchronous recruitment that is characteristic of reinnervation.
- *Group 3 - Rabbits 5 and 6 - Crotoxin at 1.5 mg:* nonspecific signs of irritability persist and the present motor units showed a reduced number.
- *Group 4 - Rabbits 7 and 8 - Botulinum Toxin Type A (2U):* signs of irritability were no longer present and motor units showed normal amplitude and duration during contraction. The interference pattern was still incomplete, but long polyphasics (asynchrony) were not found, which suggests a complete regeneration.
- *Group 5 - Rabbits 9 and 10 - Crotoxin at 15 mg:* normal potentials with signs of recent reinnervation in rabbit 10.
- *Group 6 - Rabbits 11 and 12 - 150 mg e 75 mg:* potentials were normal during contraction, but with reduced motor units, mainly in rabbit 11.

Table 6 shows the findings of electromyographic examinations accomplished fifty-eight days after toxin applications in rabbits 1 to 8, and fifty-one days after in rabbits 9 to 12:

Superior straight Muscle of rabbit nº	Myoelectric activity														
	Repose						Moderate Contraction Maximum Action potential						Contraction		
	Increased Insertion activity	Fibrillation	Positive waves	Bizarre discharge	Fasciculation	Repose silence	P.A.U.M.N *	Low amplitude potential	Giant potentials	Long polyphasics	Short polyphasics	Absence of potentials	Normal interference pattern	Incomplete interference pattern	Motor unit interference pattern
1 Crtx 0.015µg								X						X	
2 Crtx 0.015 µg								X							
3 Crtx 0.15 µg								X							
4 Crtx 0.15 µg								X							
5 Crtx 1.5 µg								X							
6 Crtx 1.5 µg								X							
7 Botox ® 2 unidades								X							
8 Botox ® 2 unidades								X					X		
9 Crtx 15 µg								X					X		
10 Crtx 15 µg								X							
11 Crtx 150 µg								X							
12 Crtx 75 µg				X				X							

** Normal action potentials of motor units.*

The examinations were practically normal, although recruitment in rabbit 2 was incomplete (which might be due to an artifact as the previous examination had been normal). Nonspecific signs of irritability remained in rabbit 12, with no other alterations.

EXAMPLE 4: ANATOMO-PATHOLOGICAL STUDY

Animals were sacrificed by means of intracardiac injections of bupivacaine chloride at 0.75% (Cristalia Produtos Qufmicos Farmaceuticos Ltda., Itapira, SP, Brazil) after the last electromyographic examination was accomplished on October 7, 2000. The right orbit of odd-number rabbits - each representing a group studied - was carefully exenterated. The specimens were put in paraformaldehyde at 4% (Formax Produtos Farmaceuticos, Divinópolis, MG, Brazil) and processed for histopathological examination.

The right orbit of six rabbits - a sample of each group - was exenterated for histological examination seventy days after crotoxin or botulinum toxin type A application. In all groups, the muscular changes found in superior rectus muscle were located at 4-5 cm posterior to scleral insertion. These changes were focal, i.e., observed only on the toxin application spot (figures 47 and 48).

- *Group 1 - Rabbit 1:* a small discreet endomysial fibrosis was observed in the area crotoxin was applied.
- *Group 2 - Rabbit 3:* the discreet endomysial fibrosis observed is more evident than that of group 1.
- *Group 3 - Rabbit 5:* the lesions verified were localized and a normal muscle area could be seen near the crotoxin injection. A discreet chronic inflammation could be observed, which was characterized by a lymphocytic and histiocitary mononuclear infiltrate. Discreet myophagocytosis and edema with endomysial fibrosis were also observed. There were fibers with degeneration (vacuolated) and signs of muscular regeneration (centralization of nucleuses).
- *Group 4 - Rabbit 7:* the anatomo-pathologic changes were similar to those found for group 3. Rabbits 7 and 8 of group 4 received two units of botulinum toxin type A.
- *Group 5 - Rabbit 9:* in this group, changes were more discreet than those observed for rabbit 11 (Group 4), more significant, however, than those for groups 3 and 4.
- *Group 6 - Rabbit 11:* signs of degeneration of muscular fibers were more intense than those observed for Group 5 and necrosis signs were not observed. Signs of discreet myophagocytosis, endomysial fibrosis and chronic inflammatory infiltrate were clearly shown.

As for side effects, ptosis practically affected all rabbits one day after the toxin application, except for rabbit 4. The pathology was discreet, however, and rabbits 1, 2, 3, 5 and 6 were better after the second day. Ptosis remained for longer in rabbit 8 (two weeks), who had received botulinum toxin type A. As for the experiment of SCOTT, ROSENBAUM and COLLINS (1973), ptosis had been affecting for up to six weeks after the application in the monkey who had received the highest dose of botulinum toxin type A. The dosage used in the animal presenting ptosis for six weeks in SCOTT *et al.* experiment was higher than the one used in our experiment. Additionally, a 100 ml volume was applied to all animals in our study and it reached up to 500 ml in SCOTT,

ROSENBAUM and COLLINS' (1973) experiment, a factor facilitating the substance diffusion in adjacent tissues. The animals who had received A-bungarotoxin (snake venom) in SCOTT's experiment presented ptosis and were better in two or three days.

The conjunctival hyperemia observed after application was discreet and did not last for more than 48 hours in rabbits 1, 2, 3 and 4 (who received smaller doses of crotoxin) and in rabbits 7 and 8 (who received two units of botulinum toxin type A). Rabbits 5 and 6 - who received 1.5 mg of crotoxin - showed a moderate reaction and a gradual recovery occurred within seven days. SCOTT, ROSENBAUM and COLLINS (1973) also observed a discreet reaction in animals submitted to A-bungarotoxin and botulinum toxin Who were better one day after. Rabbits 9, 10, 11 and 12 presented a more accentuated reaction with hyperemia and purulent secretion, which were spontaneously better without any topic medication in approximately seven days. In addition to conjunctival reaction, rabbit 11 presented corneal leukoma, which was in effect until the conclusion of the experiment.

EXAMPLE 5: EXPERIMENTS IN DOGS

Studies in other animal models, as dogs, are essencials to prove the ability to induce transitory extraocular muscle paralysis before applying crotoxin in humans with muscular distonias. These studies also are relevant to avaliate side effects and also to be a comparative data with botulinum toxin A.

Eight dogs without defined bred, aging between 12 and 24 months, weighting approximately 10 kg, were alleatory divided in two groups containing four animals each. The groups received the following treatment:

	Crotoxin	Botulinium Toxin A
Group I	10 units	
Group II		10 units

The toxins administration occurred at the same day and it was performed in the triceps and gastrocnemius muscles by using a neddle-electrode 37 mm X 27 G. The electromiography was performed after five days and subsequently after 30 and 60 days.

One animal of each group was sacrified in order to perfom the anatomo-pathological that was done 10 days after the injection and subsequently after 30 and 60 days. To the sacrifice, it was used 200mg/kg/IV sodium tiophental. The animals injected were observed in order to find any side effect reactions.

Crotoxin induced a transitory paralysis in the musculature without causing any significant side effects. These results show that crotoxin is able to be applied in the treatment of muscle dystonias as an alternative for treatment with botulinium toxin A. Besides, crotoxin presents lower cost than botulinium toxin A.

EXAMPLE 6: ELECTROMYOGRAPHIC REGISTERS OF TRICEPS AND GASTROCNEMICUS MUSCLES

It was performed electromyographic register (EMG) of 02 triceps muscles and 02 gastronemius muscles of all eight animals. So, a total of 32 muscles were analized. Four animals received crotoxin injection and the other four, the injectiong of botulinium toxin A. The toxin administration occured after the EMG register.

Figures 1 to 4 represent the EMG register before crotoxin injection. Figures 5 to 8 represent the EMG register before botulinium toxin A.

Figures 9 to 12 show the EMG register after crotoxin injection. Figures 13 to 16 show the EMG register after botulinium toxin A.

The animals did not show any systemic alteration or loss of apetite after the injections. They also did not present any signal of ambulation. The first control EMG was performed 5 days after the injections.

After the toxin injections in the respective animal group, all muscles presented significant modifications on the EMG traces. A decrease of the action potential was evident when compared to normal potencials (control groups, no toxin applied). Polymorfous potencials, sparses, positive waves and fibrilations were found in both groups.

EXAMPLE 7: EXPERIMENTS IN HUMANS

Three humans, who would be submitted to eyeball evisceration for other reasons, were invited to participate in the study by the time crotoxin application in humans was about to start. They have freely signed a term of consent, after being thoroughly informed of all implications. The study had been approved by the Ethics Committee of UFMG (COEP-UFMG). Then, these three individuals went to the Hospital Sao Geraldo so as to have their crotoxin application follow up. Two units of crotoxin were applied, corresponding to 2 LD-50, in the medial rectus muscle of the atrophied eye. One patient only presented a discreet conjunctival hyperemia in the application spot, which was spontaneously better within two days. No systemic symptomatology related to crotoxin application was observed during the postoperative period. Expenses related to the patients' hospital stay were incurred by the researchers.

Following these good results with no adverse effects, it was decided to start an assessment of the strabismic patients in sequence of the botulinum toxin procedures.

In his first study (SCOTT, A.B. Botulinum toxin injection into extraocular muscles as an alternative to strabismus surgery. Ophthalmology, v.87, p.1044-1049, 1980), SCOTT performed 67 botulinum toxin applications in 19 patients. The first initial doses producing effects were 6.25 x 10^{-5} e 3.12 x 10^{-4} micrograms in a 0.1 ml solution. The latter was then repeated or increased depending on the response. The LD-50 of the injected toxin was of 4.3 x 10^{-4}. All applications with dosage up to 6.25 x 10^{-5} required other injections or the treatment was taken as "inadequate".

When the dosage of 3.12 x 10^{-4} in 0.1 ml solution was applied, the response of four out of *ten* applications was considered as adequate. As for the dosage of 1.56 x 10^{-3}, i.e., 3.62 LD-50, two out of four applications were considered adequate. The maximum applied dosage was of 7.8 x 10^{-3}, corresponding to 18 LD-50.

According to the table in which the author defined the botulinum toxin effects, no patient showed results exceeding 60 days in duration. In order to make the analysis and comparison easier, this study results are described just in the same way as Dr. Scott has used in his initial article on botulinum toxin. Twelve applications were performed in nine strabismic patients in our crotoxin study.

Table 7: Patients submitted to crotoxin application (1 U = 1 DL 50)

Patient	Age	DP	Crotoxin	Reduction	Ocular	Effect	Adverse effect

		deviation	dose applied	of DP deviation	movement limitation in the range of action of applied muscle	duration	
01	52	HoTE 35	2 U	15	-2/-4	3 months	none
02	21	ET 50	2 U	20	-1/-4	1 month	none
03	37	XT 20	2 U	zero	There was none	----------	Discreet ache on the application spot for 3 days
04	18	ET 65	2 U	20	-1/-4	1 month	A twitching on the spot on the application day
05	35	ET 45	2 U	27	-1/-4	1 month	none
05	35	ET 45	2 U	25	-1/-4	1 month	none
06	43	ET 95	2 U	15	There was none	1 month	none
07	19	ET 10	2 U	20	There was none	2 months	none
07	19	ET 10	4 U	55	-4/-4	3 months	none
08	49	ET 35 a 40	2 U	zero	There was none	--------	Discreet conjunctival hyperemia on the application spot
08	49	ET 35 a 40	4 U	20	-1/-4	2 months	A twitching on the spot for two days
09	44	ET 20	5 U	16	-1/-4	3 months	none

No systemic effect was observed in the patients submitted to crotoxin application in the extraocular musculature for the strabismus treatment. No changes in vision or in any other ocular structure related to the toxin application were observed. The adverse effects observed were as follows: discreet conjunctival hyperemia in one patient and symptoms of pain or twitching on the application spot in three patients. No patient received any local or systemic medication after the toxin application. Adverse effects spontaneously vanished in all cases. Changes in ocular deviation were not observed in 2 patients after 2 U of crotoxin were applied. As expected, limited ocular movement in the range of action of applied muscle was observed in 8 out of 12 applications. This effect varied from 1 to -4, in a scale ranging from 25% to 100%, and in all cases there was a progressive and complete recovery of ocular movements after a variable period of time. The ocular deviation reduction after 2 U crotoxin application (9 applications) amounted to an average of 15.7 prismatic diopters. As for dosage of 4 U (2 applications), the prismatic diopters amounted to an average of 37.5. As for the only 5 U crotoxin application, 16 prismatic diopters in ocular deviation were obtained. When ocular alignment change did appear after crotoxin application, it lasted for 1 to 3 months. In his study published in 1980, Scott observed - using botulinum toxin - a deviation reduction of up to 30 prismatic diopters with an effect duration of up *to 2* months.

After this stage accomplished with strabismic patients, we have decided to apply the drug in patients with essential blepharospasm. Three patients were invited to participate in the study and, after their consent, crotoxin was applied to them. All these patient had previously been submitted to botulinum toxin application. The first patient had been submitted to 10 botulinum toxin applications and there was no significant improvement after the crotoxin applications. The two other patients observed improved hemifacial spasms, which intermittently came back two months after the application. Two patients reported the presence of a discreet pain on the application spot, which disappeared within two days after. One of the patients reported that no difference crotoxin effect and that of botulinum toxin was observed.

After these results, the ideal dosage of crotoxin to be applied in extraocular muscles is 5 U diluted to 0.1 ml. In the applications of 4 U with 0.2 ml and for of 5 U with 0.25 ml were observed eased drug extravasation, which dilutes the desired action and increases the possibility of complications.

Based on the results observed in the present patent experiment, crotoxin showed to be a therapeutic option for several medical areas that currently use the botulinum toxin. A national drug would be so in use, saving foreign currencies to be used for botulinum toxin imports.

CLAIMS

1. "USE OF PHARMACEUTICAL COMPOUND CONTAINING CROTOXIN" characterized by being used in preparing of a drug for treating muscle dystonias.
2. "USE OF PHARMACEUTICAL COMPOUND CONTAINING CROTOXIN" in accordance with claim 1, characterized by being used in preparing of a drug for treating preferably ophthalmologic muscle dystonias, such as comitant strabismus (esotropia, exotropia or hypertropia), incomitant strabismus (paralytic), Duane syndrom, restrictive or myogenic strabismus, blepharospams, nystagmus, palpebral retraction in hyperthyroidism or therapeutic ptosis for corneal protection;
3. "USE OF PHARMACEUTICAL COMPOUND CONTAINING CROTOXIN" in accordance with claim 1, characterized by being used in preparing of a drug for treating pathological muscular conditions in neurology, such as tremors, muscular spasticity following a cerebrovascular accident, cerebral paralysis, skull traumatisms and traumatisms of the vertebral column;
4. "USE OF PHARMACEUTICAL COMPOUND CONTAINING CROTOXIN" in accordance with claim 1, characterized by being used in preparing of a drug for treating pathological muscular conditions in dentistry, such as bruxism and temporomandibular articulation dysfunctions;
5. "USE OF PHARMACEUTICAL COMPOUND CONTAINING CROTOXIN" in accordance with claim 1, characterized by being used in preparing of a drug for treating pathological muscular conditions in surgery and coloproctology;
6. "USE OF PHARMACEUTICAL COMPOUND CONTAINING CROTOXIN" in accordance with claim 1, characterized by being used in preparing of a drug for treating wrinkles and/or cosmetic problems;
7. "USE OF PHARMACEUTICAL COMPOUND CONTAINING CROTOXIN" in accordance with claim 1, characterized by being used in preparing of a drug for treating muscle disturbances associated with gland hypersecretions, such as plantar hyperhidrosis, axillary hyperhidrosis and/or gustatory hyperhidrosis;
8. "USE OF PHARMACEUTICAL COMPOUND CONTAINING CROTOXIN" in accordance with claims from 1 to 8, characterized by the administration of the pharmaceutical compound be performed by intramuscular injection.

FIGURES

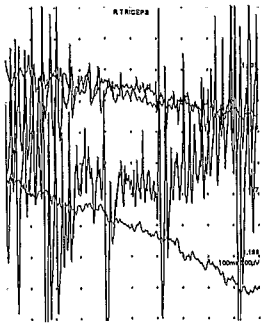
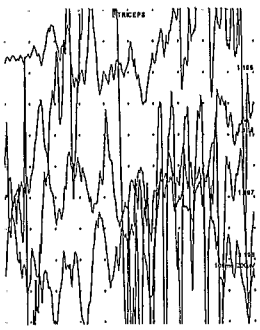


Figure 1



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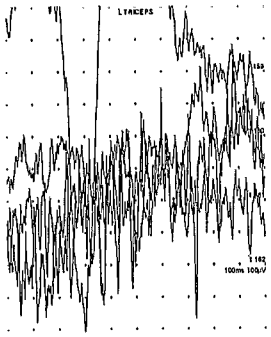

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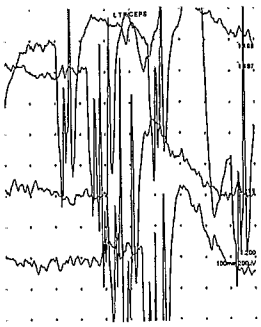

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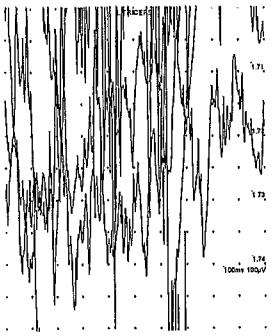

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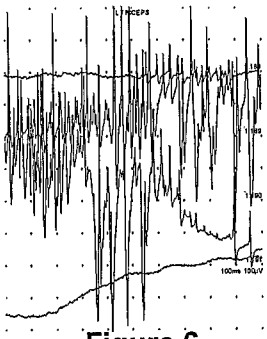

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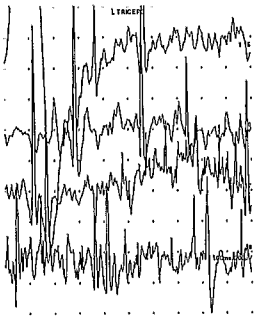

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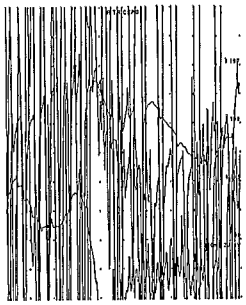

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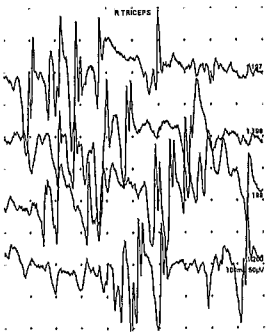


Figure 9

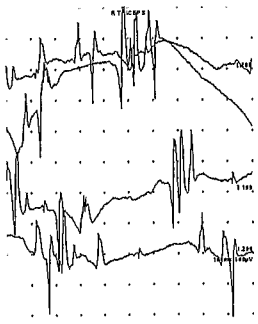
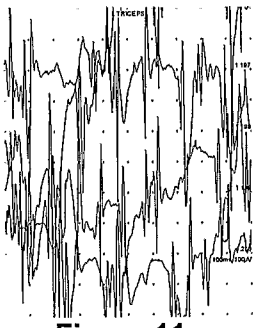


Figure 10



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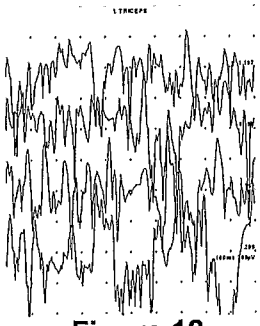


Figure 12

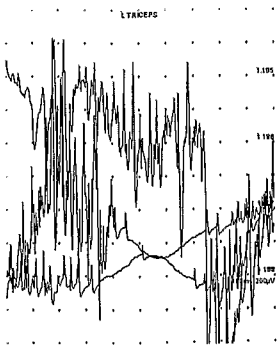
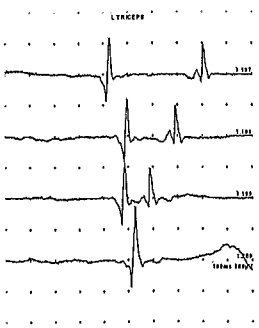


Figure 13



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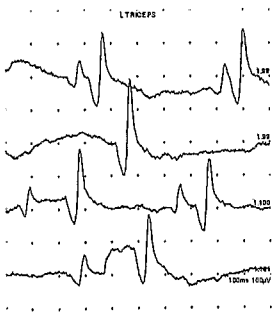

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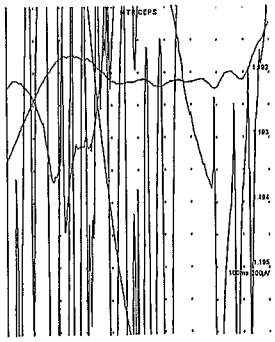

Figure 16