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Original Article

Effect of botulinum neurotoxin type A (BoNTA) on the morphology and viability of 3T3 murine fibroblasts

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Abstract: Aim: BoNTA is used in the treatment of ophthalmological disorders, muscular hyperactivity and pain. In recent years it has been described that BoNTA reduces cellular viability and induces apoptosis in prostate cells lines. Studies about the effect of BoNTA are no well known. There have been studies about the effect of BoNTA on the expression levels of collagenase in fibroblasts, but not on its morphological impact on these cells. The aim of this study was to determine the effect of BoNTA on the morphology and viability of the 3T3 fibroblast cell line. Material and methods: The 3T3 fibroblast cell line was cultured and the experimental group received 10 U BoNTA added to a 0.9% sterile saline solution in a reconstituted vial. The control group received saline solution only. Cultured cells were observed and photographed at 5, 10, 15 and 20 h. Cell viability was evaluated by means of the trypan blue test, and cell proliferation with the Proliferation Assay kit (PROMEGA). Results: The application of BoNTA to 3T3 fibroblast cells induced morphological changes, such as a loss of normal fibroblast morphology. Additionally, we observed the cytoplasmic retraction and spread phenomena. The nuclei showed other important changes with Giemsa staining. Conclusion: The results indicate that BoNTA induced a loss of spindle form, increase in cytoplasmic vesicles, and the presence of nuclear vesicles (compacted chromatin surrounded by a nuclear envelope). This suggests an apoptotic process and decreased cell viability. Further studies are needed to explore the mechanisms of these alterations.

Keywords: Botulinum neurotoxin, fibroblasts, morphology, apoptosis, genotoxicity, cell viability

Introduction

Botulism, first reported over a century ago, is caused by botulinum neurotoxins produced by the Gram positive bacterium Clostridium botulinum. This bacterium has seven immunological serotypes (A-G) that can cause botulism. The biological actions of botulinum neurotoxins (BoNT) have been widely studied.

BoNT inhibits the release of a neurotransmitter-acetylcholine-in motor nerve endings, thus blocking nerve function. This characteristic has been exploited in therapeutic applications to reduce muscle hyperactivity, involuntary muscle spasm and contractions [1]. Some physicians have reported a face-lifting effect due to increased collagen synthesis after an intradermal injection of BoNT type A (BoNTA) to the mid and lower face [2-4]. However, the effect of an intradermal injection of BoNTA is still not clear.

One study showed that BoNTA can reduce viability of the LNCaP cell line by triggering apoptosis [5]. Another study explored the possible effect of BoNTA on fibroblasts, since it has been reported that 3T3 fibroblasts express the synaptic vesicle protein type 2 isoform A (SV2A), which is the receptor for BoNTA [5-12]. Nevertheless, this study did not find any negative effects of BoNTA on human dermal fibroblasts [4].

The possibility that BoNTA applications could induce cell damage in fibroblasts through modification in traffic and signaling processes, cytoskeleton inhibition, mitosis deactivation, and induction of apoptosis requires further

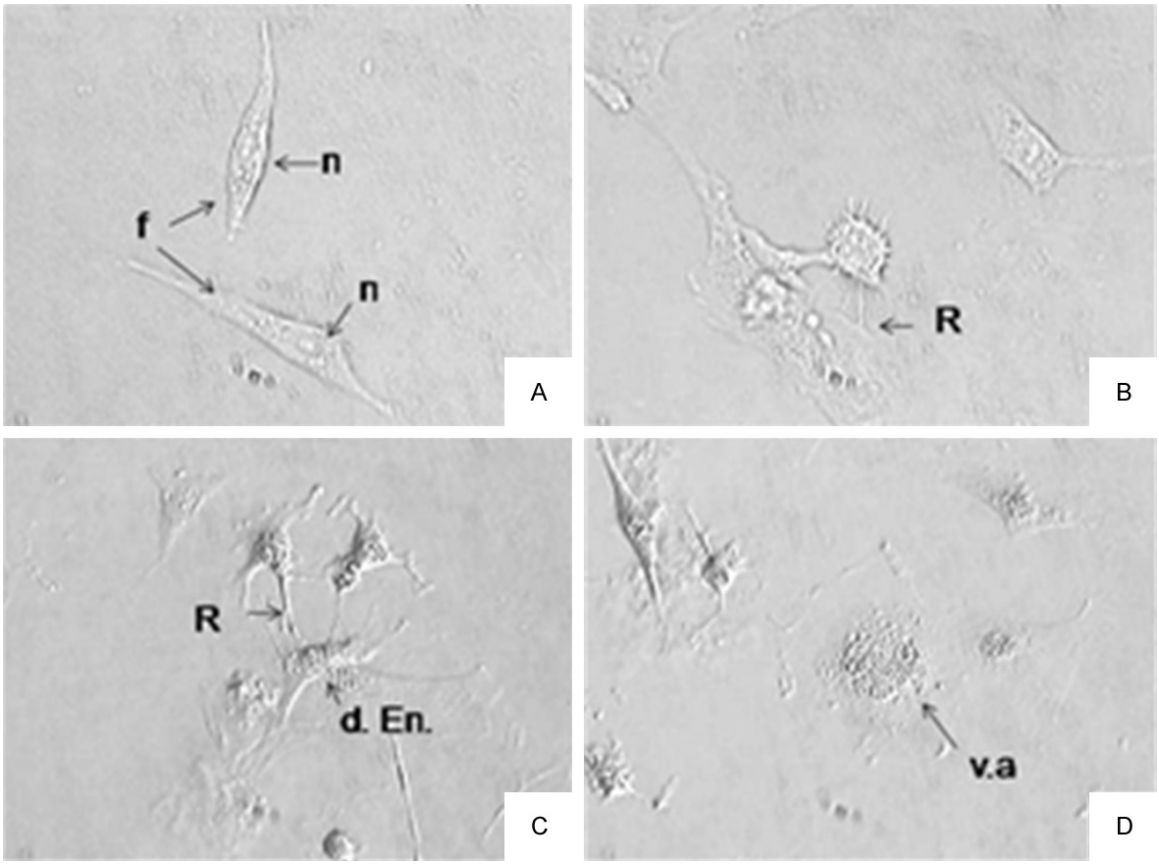

Figure 1. Effect of BoNTA (10 U) on fibroblast morphology (20×). (A) Interaction at 5 h, (B) 10 h, (C) 15 h and (D) 20 h. f= fibroblast, R= retraction, d. En.= degranulation, v.a.= apoptotic vesicle.

research. The aim of the present study was to determine the effect of BoNTA on the morphology and viability of the 3T3 fibroblast cell line.

Material and methods

Experimental design

This was a longitudinal, comparative and experimental study. The 3T3 fibroblast cell line was cultured and treated with BoNTA or a saline solution. Cell viability and cell proliferation were then microscopically evaluated.

Cell line and cell culture

Murine fibroblasts of the 3T3 cell line were cultured in MEM (GIBCO) medium with 5% Bovine Fetal Serum (GIBCO) and an antibiotic (SIGMA). All culture plates were incubated with 5% CO_2 at 37°C since to reach 80% confluence.

Treatment

Once cultures reached confluence (considered time zero), all cells were washed with PBS. The culture plates were then divided in the treated and control groups. The treated group received 10 U BoNTA added to a 0.9% sterile saline solution in a reconstituted vial (4.8 ng/100 U, Allergan, Inc.). The control group received saline solution only. Each assay was performed in triplicate.

Microscopy

Cultured cells were observed and photographed at 5, 10, 15 and 20 h after the application of BoNTA. The morphology of cells was observed on slides with an inverted Olympus MIC-D Digital Microscope, using phase contrast, an imaging acquisition system, and a 40x objective lens. Cells cultured for 10 and 20 h were stained with Giemsa to observe the nuclei. Cell viability was evaluated by means of the trypan blue test, and cell proliferation with the Proliferation Assay kit (PROMEGA).

Statistical analysis

The percentage of cells with a different morphology was determined for each observed

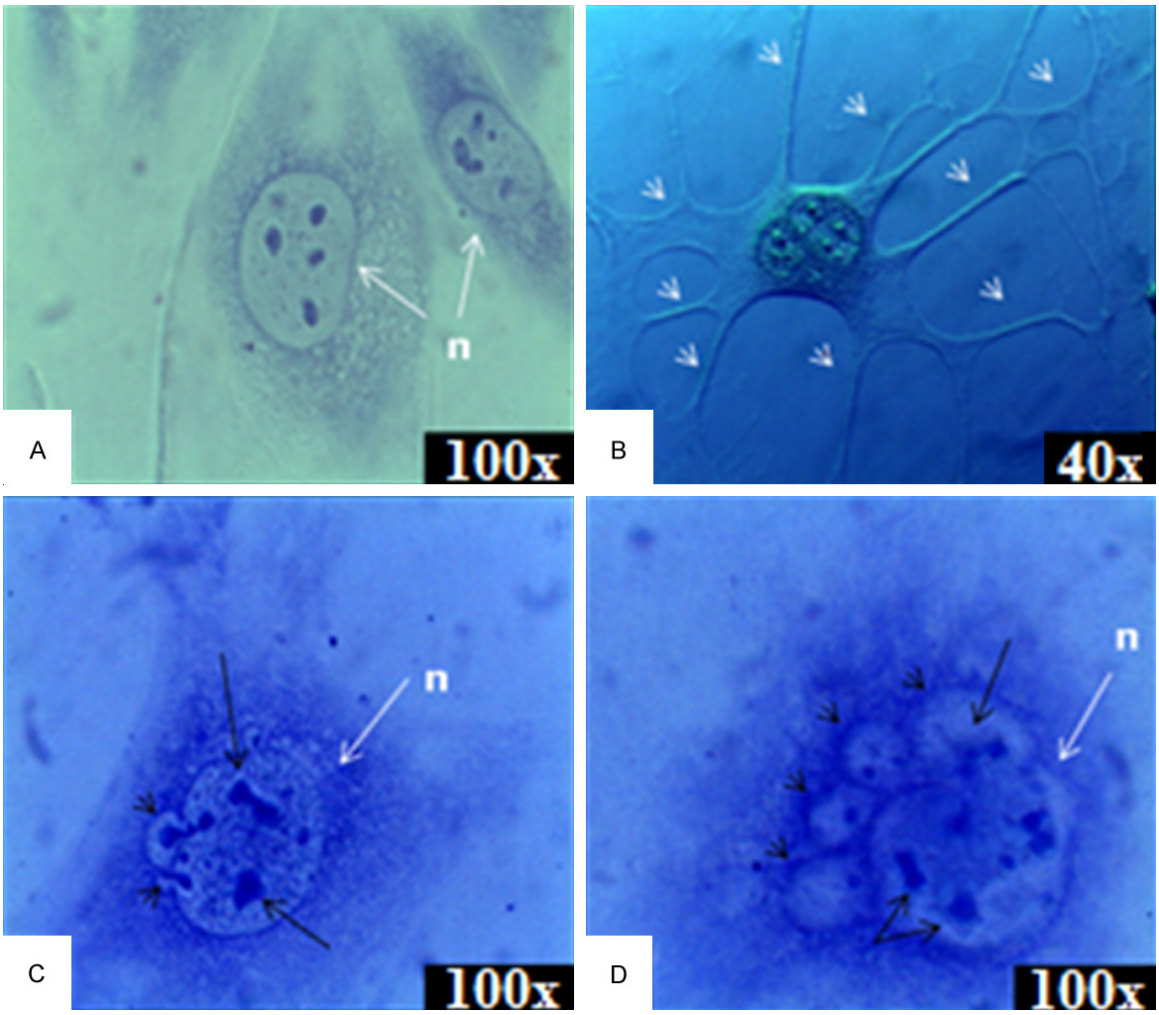

Figure 2. Effect of BoNTA (10U) on the fibroblast cytoplasm and nucleus, shown by Giemsa staining. (A) Untreated fibroblast (100×), (B) cytoplasmic retraction (arrowhead) at 10 h of interaction (40×), and (C and D) formation of the nuclear envelope vesicle with condensed chromatin inside at 15 and 20 h, respectively. n= nucleus.

field, followed by calculating the mean and standard deviation in relation to cell morphology, viability and proliferation. The Mann Whitney U test was used to analyze these parameters. Analysis was performed with SPSS v17 for Windows XP (SPSS, UK, Ltd, Woking, UK). $P<0.05$ was regarded as significant.

Results

The application of BoNTA to 3T3 fibroblast cells induced morphological changes (**Figure 1**), such as a loss of normal fibroblast morphology characterized by a branched cytoplasm surrounding an elliptical, speckled nucleus having one or two nucleoli (**Figure 1A**). These changes were observed at 10 h of interaction (**Figure 1B-D**). Additionally, we observed the cytoplasmic retraction and spread phenomena, the formation of cytoplasmic extensions (microspikes and microvilli), and internal reorganization with abundant cytoplasmic granules and vesicles. The nuclei showed other important changes with Giemsa staining (**Figure 2**). The most important changes were observed at 10-15 h (**Figure 2B-D**), involving nuclear-membrane vesicle formation with compacted chromatin inside (**Figure 2C**, **2D**). At 20 h, the BoNTA application increased nuclear vesicle formation and cytoplasmic degradation in 75% of fibroblasts, accompanied by depleted cell viability ($P=0.04$).

Discussion

As has been shown previously in muscle tissue, BoNTA injections induced morphological chang-

es in the present study, including muscle fiber atrophy, disorganization of the muscle fiber structure, extension of nerve terminal sprouts, and formation of new neuromuscular junctions [13]. In 3T3 fibroblasts we found that 10 U of BoNTA induced morphological changes, including a loss of spindle form and cytoplasmic retraction. Functional alterations can be related to different adaptive phenomena [1, 14].

Other studies have reported inhibition of motility and mitosis in cells that are exposed to BoNTA [15] due to the disassembly of actin filaments, alteration of focal adhesions, and rounding of cells. It has also been reported that botulinum toxin may induce a proliferative response by acute and chronic mitosis in the muscle tissue of rat [16]. All of these events can be associated with the present findings: cytoplasmic disruption, cytotoxic cell damage and cell death [17-19].

BoNTA has a dose-dependent toxic action that involves the mechanism of chemodenervation, inhibiting acetylcholine release and leading to functional denervation of striated muscle. This results in muscle fiber atrophy and subsequent clinical flaccid paralysis [4]. BoNTA could be internalized in cells by binding to the SV2 receptor and in this way inhibit the liberation of vesicles, which would allow for their accumulation and consequently modification of intracellular traffic [7]. These changes could promote the secretion of typical healing substances, such as tropoelastine (elastic fiber), tropocollagen (collagen fiber type I and III or reticular fiber) and amorphous substances [20-22].

However, we found that cell viability decreased after treatment with BoNTA, suggesting an adaptive response involving BoNTA-linked cell damage. Such damage is evidenced by the presence of nuclear vesicles with compacted chromatin and an increased formation of micronuclei, which could be associated with genocytotoxicity [23] by chromosomal damage and instability. Apoptosis-linked mechanisms may be involved in the condensation of chromatin inside nuclear envelope vesicles as well as in cytoplasmic retraction [24].

In summary, by using a specific dosage and different exposure times of BoNTA, the current findings show a damaging effect of this toxin on the morphology of fibroblasts. However, other studies are required to reveal the molecular mechanisms at play.

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Disclosure of conflict of interest

None.

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