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Mitophagy in Botulinum Toxin Type A-Induced Muscle Atrophy

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ABSTRACT

Objective: Botulinum toxin type A (BTXA) is widely used in oral and maxillofacial surgery to treat masseter hypertrophy and bruxism, inducing transient masseter atrophy, but the underlying mechanisms remain unclear. Mitophagy, essential for muscle fiber homeostasis, plays a critical role in muscle atrophy. This study aims to investigate whether mitophagy mediates BTXA-induced masseter muscle atrophy.

Methods: Rats received BTXA injections into masseter for 2 and 8 weeks. Muscle fiber composition was assessed via histology and immunofluorescence. Mitophagy markers (LC3-II, p62, beclin-1, Tomm20) were quantified by western blot. Mitochondrial function was evaluated via ATP content and mitochondrial DNA (mtDNA) copy number.

Results: BTXA injection led to transient masseter muscle atrophy. During this process, the proportion of type IIA muscle fibers significantly increased, while the proportion of type IIB fibers decreased. Additionally, at 2 weeks post-BTXA injection, the expression levels of LC3-II, p62, and beclin-1 were notably upregulated, whereas Tomm20 expression was downregulated. Furthermore, a significant reduction in ATP content and mtDNA copy number was observed at the same time point, indicating impaired mitochondrial function.

Conclusion: These findings suggest that mitophagy plays a crucial role in BTXA-induced masseter muscle atrophy, providing new insights into the mechanisms underlying BTXA treatment.

1 | Introduction

Botulinum toxin type A (BTXA) is a neurotoxin produced by the anaerobic bacterium *Clostridium botulinum*. BTXA interferes with the function of synaptosomal-associated protein of 25 kDa (SNAP-25), which is essential for synaptic vesicle fusion, thereby preventing the release of acetylcholine (Choudhury et al. 2021). BTXA is increasingly used in oral and maxillofacial medicine for esthetic enhancement, treatment of movement disorders, and pain management. Esthetically, it is commonly applied to address masseter hypertrophy and gummy smile, improving facial contour and smile appearance (Kassir et al. 2024). For movement disorders, it serves as an effective treatment for hemifacial spasm (Maytharakcheep and Bhidayasiri 2025) and bruxism (Yacoub et al. 2025), significantly alleviating muscle spasms and abnormal activity. In pain management, BTXA has been shown to relieve

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chronic orofacial pain associated with trigeminal neuralgia and temporomandibular joint disorders (Sharav et al. 2025). Notably, most of these therapies involve BTXA injection into the masticatory muscles, particularly the masseter, to achieve therapeutic effects. While research has shown that BTXA can induce transient atrophy of the masseter muscle, the precise mechanisms underlying this effect remain incompletely understood.

Mitophagy is a specialized form of autophagy, wherein damaged mitochondria are selectively eliminated by the autophagy-lysosome system. Extensive research has demonstrated that mitophagy is essential for maintaining muscle fiber homeostasis under both physiological and pathological conditions, and plays a key role in supporting skeletal muscle plasticity (Borgia et al. 2017; Sato et al. 2018). In skeletal muscle tissue, mitophagy activity increases in response to pathological factors such as aging, denervation, and fasting, leading to enhanced protein degradation (Lei et al. 2024). However, it remains unclear whether mitophagy is involved in the masseter muscle atrophy induced by BTXA.

Previous studies have shown that BTXA can temporarily increase the number of autophagosomes in the submandibular glands of rats (Xie et al. 2019), and also can promote autophagy in human skin keloid fibroblasts (Hou et al. 2019). These findings indicate that BTXA can activate autophagy; however, it remains unclear whether BTXA induces mitophagy in the masseter muscle. Therefore, this study aimed to investigate the role of mitophagy in BTXA-induced masseter muscle atrophy.

2 | Materials and Methods

2.1 | Animals

A total of 24 male Sprague–Dawley rats (7–8 weeks old, 220–250 g) were obtained from the Laboratory Animal Service Center, Peking University Health Science Center. The rats were randomly assigned to two groups: control ($n=8$) and BTXA ($n=16$). In the BTXA group, eight rats were sacrificed 2 weeks after injection and the remaining eight at 8 weeks. The animal experiments were approved by the Ethics Committee of Animal Research, Peking University Health Science Center (No. DLASBD0642). All procedures were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996).

The rats were anesthetized with isoflurane by inhalation. To ensure precise injection into the masseter muscle, a skin incision was made to expose the left masseter muscle. Following exposure, the control group received multipoint injections of 0.3 mL saline, while the BTXA group was administered 6 U of BTXA (Lanzhou Biological Co., China) dissolved in 0.3 mL saline through intramuscular injection into the left masseter. The incisions were subsequently closed using 4–0 polypropylene sutures. Tissue samples were collected at 2 and 8 weeks post-injection for subsequent analysis. The rats were euthanized using CO_2 inhalation.

2.2 | mtDNA Copy Number

The total DNA was extracted using a DNA isolation kit (TIANGEN, China) according to the manufacturer's instructions. The mtDNA copy number was quantified by real-time PCR. Primers targeting the Cytb gene of the mitochondrial genome were used for mtDNA quantification, with β-actin, a nuclear DNA reference gene, serving as the control. The sequences of the primers are listed in Table 1.

2.3 | ATP Measurements

ATP content in masseter muscle tissues was measured using the ATP Assay Kit (Beyotime, China), following the manufacturer's instructions. All experiments were conducted in triplicate, and protein concentrations were measured prior to the ATP assays.

2.4 | Transmission Electron Microcopy

Masseter tissues were preserved in a fixative solution containing 2% paraformaldehyde and 1.25% glutaraldehyde, followed by staining with uranyl acetate and lead citrate. The prepared specimens were then analyzed using a transmission electron microscope (H-7000, HITACHI, Japan).

2.5 | Western Blot

Total protein extracts from the masseter were quantified using the bicinchoninic acid assay. Proteins (20–40 μg) were resolved by 10% or 12% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk to minimize nonspecific antibody interactions, followed by overnight incubation with primary antibodies at 4°C. HRP-conjugated secondary antibodies were applied for 2 h at room temperature, and protein bands were visualized using an enhanced chemiluminescence reagent (Biodragon Technology, China).

2.6 | Immunofluorescence Staining

Masseter muscle was frozen by liquid nitrogen-chilled isopentane in Optimal Cutting Temperature compound and stored at −80°C. Serial cross-sections of 10 μm thickness were obtained for staining. The sections were fixed with 4% paraformaldehyde, permeabilized in 0.3% Triton X-100, blocked with 3% BSA.

TABLE 1 | Primers sequences used for mtDNA copy number.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Cyt B	GCAGCTTAACATTCCGCCCAATCA	TGTTCTACTGGTTGGCCTCCGATT
β-actin	TCGTGCGTGACATTAAAGAG	ATTGCCGATAGTGATGACCT

They were then incubated with primary antibodies specific for MyHC-β (type I fibers; DSHB BA-F8, USA), MyHC-2A (type IIa fibers; DSHB SC-71, USA), and MyHC-2B (type IIb fibers; DSHB BF-F3, USA). The slices were washed with PBS and incubated with the following secondary antibodies: Fluorescein (FITC) AffiniPure Goat Anti-Mouse IgG, Fcγ subclass 1 specific (115-095-205, Jackson ImmunoResearch, USA), Brilliant Violet 421 Goat Anti-Mouse IgG, Fcγ subclass 2b specific (115-675-207, Jackson ImmunoResearch, USA), Rhodamine Red Goat Anti-Mouse IgM, μ chain specific (115-295-020, Jackson ImmunoResearch, USA). Images were obtained on an EVOS FL auto microscope (Life Technologies, USA).

2.7 | TUNEL Staining

TUNEL Detection Kit (Elabscience, China) was used for TUNEL staining. All procedures were performed according to the manufacturer's instructions. A positive control was also included to validate the experimental results. For the positive control, we treated sections from the control group with DNase I.

2.8 | Statistical Analysis

Data are shown as mean ± SEM. Statistical differences among groups were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons. Statistical significance was set at $p < 0.05$.

3 | Results

3.1 | Effect of BTXA on Morphological Structure of Masseter Muscle

Histomorphological evaluation of masseter muscles, conducted via hematoxylin and eosin (H&E) staining, revealed a clear reduction in muscle fiber diameter 2 weeks post-BTXA injection. Additionally, notable nuclear proliferation and increased connective tissue were observed. By 8 weeks post-injection, although muscle fiber diameter had shown recovery, the tissue organization did not return to the orderly structure seen in the control group (Figure 1A). Muscle-atrophy F-Box (MAFbx)/atrogin-1 and muscle ring-finger-1 (MuRF-1) serve as biomarkers for muscle atrophy. The expression levels of atrogin-1 and MuRF-1 were assessed through western blot (Figure 1B). The results showed a significant increase in the expression of both atrogin-1 and MuRF-1 2 weeks post-injection. However, by Week 8, the expression of atrogin-1 showed a decreasing trend compared to 2 weeks, while the expression of MuRF-1 was significantly reduced (Figure 1B).

3.2 | Effect of BTXA on Muscle Fiber Type of Masseter Muscle

Muscle atrophy is often accompanied by a transformation in muscle fiber types. Our examination of the superficial masseter muscle in rats revealed a predominance of type II fibers, with an absence of type I fiber staining. In the control group, a higher proportion of type IIb fibers and a lower presence of type IIa fibers were observed. Compared to the control group, 2 weeks post-BTXA injection, there was a noticeable decrease in type IIb fibers and an increase in type IIa fibers. As the post-injection period progressed, the number of type IIb fibers increased, while the proportion of type IIa fibers declined 8 weeks after BTXA injection (Figure 2).

3.3 | Effect of BTXA on Ultrastructure of Masseter Muscle

In the control group, sarcomeres displayed uniform alignment and consistent lengths. In contrast, 2 weeks after BTXA injection, masseter muscle fibers displayed significant distortion, with distinct ultrastructural alterations. Furthermore, mitochondria showed signs of vacuolar degeneration, swelling, and disruption of cristae. Eight weeks after the injection, the alignment of muscle fibers improved slightly, but they were still somewhat distorted compared to the control group. The mitochondrial structure appeared relatively normal, with clearer morphology (Figure 3).

3.4 | Effect of BTXA on Mitophagy in Masseter Muscle

Under the transmission electron microscope, observations revealed mitochondrial structural damage following BTXA injection. Further investigation into mitochondrial function showed a reduction in mtDNA copy number (Figure 4A) and ATP content (Figure 4B) in the masseter muscle, 2 and 8 weeks after BTXA injection. Additionally, transmission electron microscopy revealed a significant accumulation of mitochondrial autophagosomes 2 weeks after BTXA injection (Figure 4C). Moreover, the expression of autophagy-related proteins was examined following BTXA injection into the masseter muscle via western blot. Notably, 2 weeks post-BTXA injection, there was an upregulation of Beclin-1, LC3-II, and p62, indicating enhanced autophagic activity. However, by 8 weeks post-injection, the expression of these proteins showed a decreasing trend (Figure 4D). TOMM20 is a key protein of the mitochondrial outer membrane, and its expression typically decreases during mitophagy. Two weeks after BTXA injection, TOMM20 expression was significantly reduced, but it returned to control levels after 8 weeks (Figure 4E). These findings suggest that mitophagy is activated during BTXA-induced masseter muscle atrophy.

In addition, no differences in the expression of the mitochondrial fusion protein OPA1, the fission protein Drp-1, or the mitochondrial biogenesis protein PGC-1α were observed 2 weeks after BTXA injection (Figure 4F). These results further suggest that BTXA-induced masseter muscle atrophy is closely associated with mitophagy.

3.5 | Effect of BTXA on Apoptosis in the Masseter Muscle

Apoptosis is another mechanism that determines cell fate. BAX is a pro-apoptotic protein, while Bcl-2 is an anti-apoptotic protein. Two weeks after BTXA injection into the masseter muscle, the expressions of BAX and Bcl-2 were both significantly

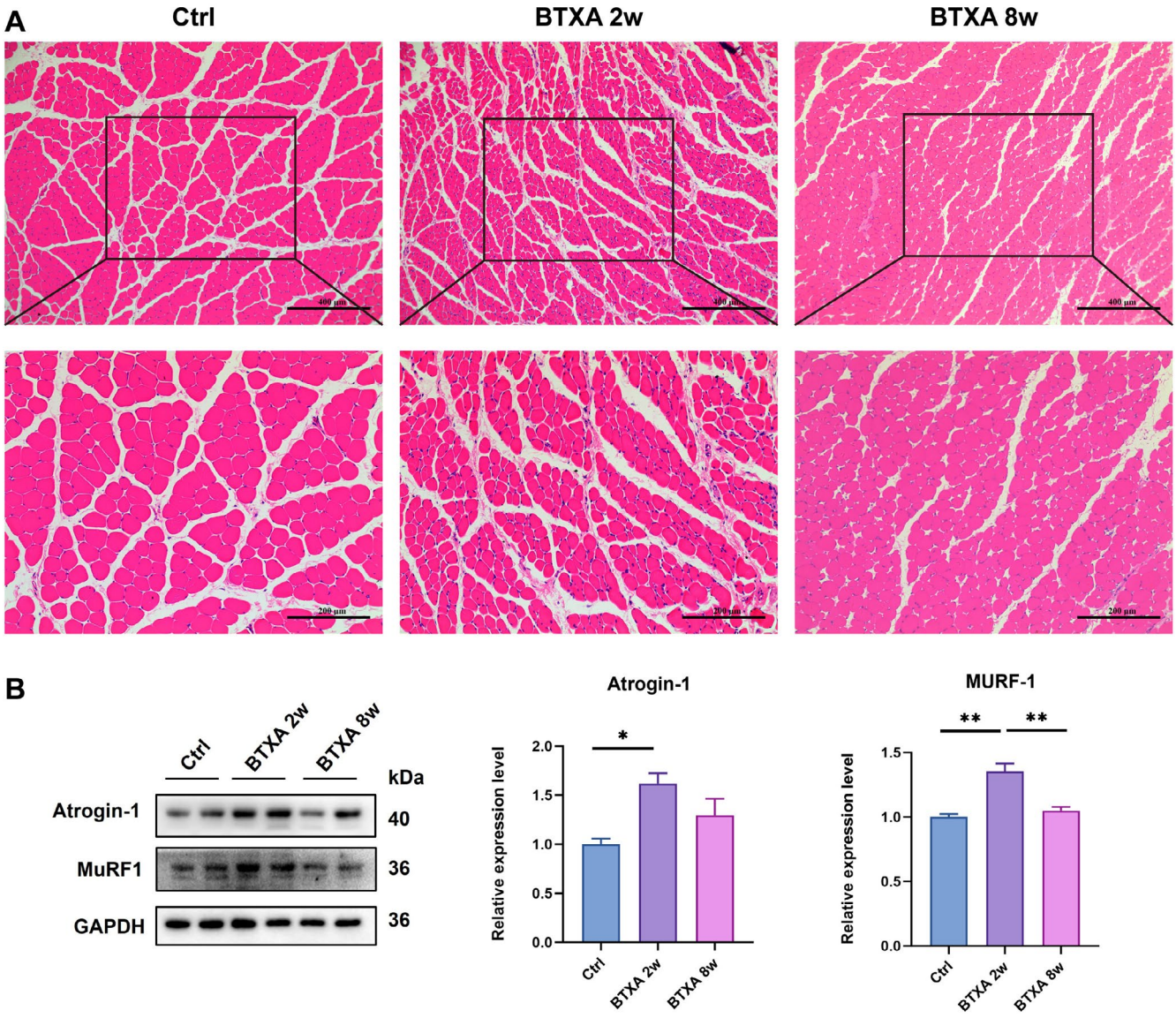

FIGURE 1 | BTXA induces masseter muscle atrophy. (A) H&E staining of the masseter muscle at 2 and 8 weeks post-BTXA injection. (B) Protein expression levels of atrophy markers Atrogin-1 and MuRF-1 at 2 and 8 weeks post-BTXA injection. Data are presented as mean ± SEM; $n = 4$, $^{*}p < 0.05$; $^{**}p < 0.01$ compared with control group.

upregulated; however, the Bcl-2/BAX ratio did not show significant differences. Eight weeks post-BTXA injection, the expression level of BAX was similar to that of the control group, but the Bcl-2/BAX ratio was significantly different from that of the BTXA 2w group (Figure 5A). In order to confirm whether BTXA induces apoptosis in the masseter muscle, we conducted TUNEL staining. The results showed no significant positive staining at either 2 or 8 weeks post-BTXA injection (Figure 5B), indicating that BTXA does not induce apoptosis in the masseter muscle. These findings further support that BTXA primarily activates autophagy in the masseter muscle.

4 | Discussion

In this study, we identified that BTXA injections temporarily induced masseter muscle atrophy. Specifically, we observed a significant increase in the proportion of type IIa muscle fibers, accompanied by a corresponding decrease in type IIb fibers as muscle atrophy progressed. Furthermore, mitophagy levels were significantly enhanced during the period of BTXA action. Notably, once the effects of BTXA started to diminish, both the distribution of muscle fiber types and the levels of mitophagy activity exhibited partial recovery. These findings suggest that BTXA may promote masseter muscle atrophy by activating mitophagy. This mechanism could provide new insights into the role of BTXA in clinical applications.

Muscle atrophy often alters the fiber-type composition of skeletal muscle, which comprises type I (slow-twitch) and type II (fast-twitch) fibers. Type II fibers include IIa, IIx, and IIb subtypes. Type IIb fibers contract rapidly and rely on glycolytic metabolism for short, high-intensity movements, whereas type IIa fibers contract more slowly but are more fatigue-resistant (Li et al. 2022; Schiaffino and Reggiani 2011). Our study's immunofluorescence staining results indicate that 2 weeks post-BTXA

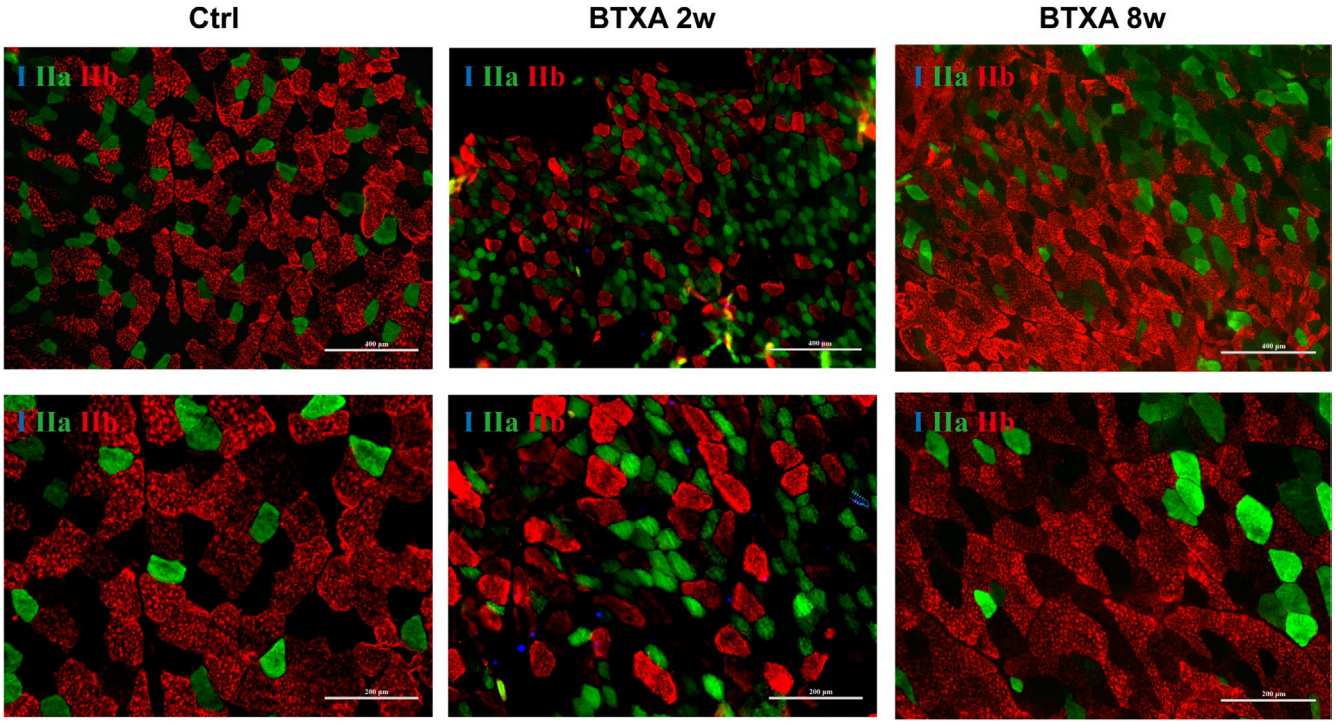

FIGURE 2 | BTXA induces transformation of muscle fiber types in the masseter muscle. Immunofluorescence staining on frozen sections of the superficial masseter muscle revealed type I (blue), IIa (green), and IIb (red) muscle fibers. Two weeks after BTXA injection, there was an increase in the proportion of type IIa fibers and a decrease in type IIb fibers.

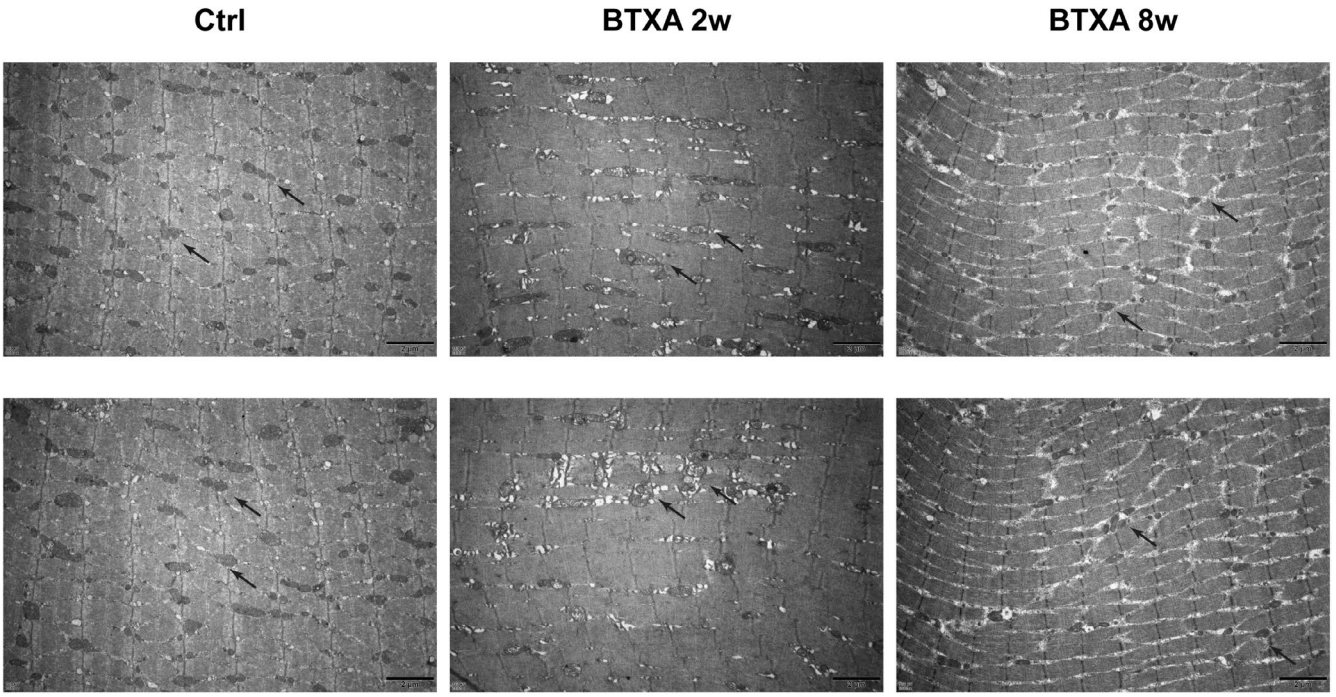

FIGURE 3 | **BTXA induces ultrastructural changes in the masseter muscle**. Microstructure of masseter muscle and mitochondria (black arrows) was observed by transmission electron microscopy.

injection, a significant decrease in type IIb fibers accompanied by an increase in type IIa fibers was observed, suggesting a possible transformation from type IIb to type IIa fibers that may enhance fatigue resistance in the masseter muscle. In our study, the increased proportion of type IIa fibers observed 2 weeks after BTXA injection into the rat masseter muscle is consistent with several previous reports. For example, a rat study with a similar design demonstrated a significant, dose-dependent increase in MyHC-IIa protein expression at Day 14 following BTXA injection (Moon et al. 2015). Another study in mice reported that,

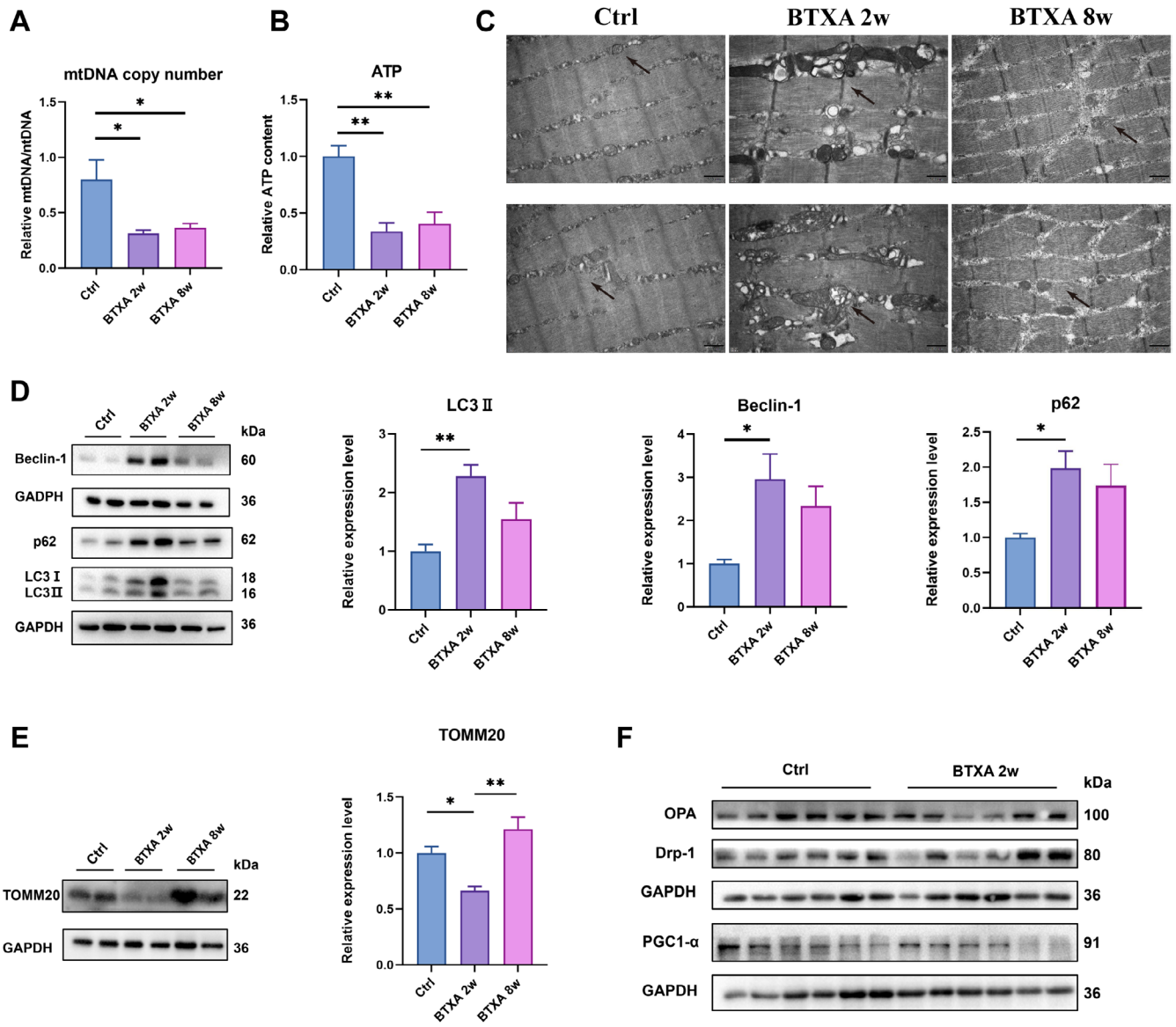

FIGURE 4 | BTXA induces mitophagy in the masseter muscle. Quantitative analysis of mtDNA copy number (A) and ATP content (B) at 2 and 8 weeks post-BTXA injection. (C) Mitochondrial autophagosomes (black arrows) was observed by transmission electron microscopy. (D) Western blot analysis of Beclin-1, LC3-II, and p62 at 2 and 8 weeks post-BTXA injection. (E) Western blot analysis of the mitochondrial outer membrane protein TOMM20 at 2 and 8 weeks post-BTXA injection. (F) Western blot analysis of OPA1, Drp-1, and PGC1-α. Data are presented as mean ± SEM; $n = 5–8$, $^{*}p < 0.05$; $^{**}p < 0.01$ compared with control group.

21 days after injection, MyHC-IIa mRNA expression was significantly upregulated, while MyHC-IIb and MyHC-IIx/d mRNA levels were downregulated (Botzenhart et al. 2016). However, another study has reported different findings. The study by Tsai et al., using immunohistochemical staining, found that 45 days after BTXA injection into both the masseter and temporalis muscles, the proportion of type IIa fibers was reduced compared to BTXA injection into the temporalis muscle alone, while no significant differences were observed in the proportions of type IIx and IIb fibers (Tsai et al. 2012). The difference is likely due to methodological variation. Our analysis was conducted at an earlier time point, likely capturing the acute response to denervation, whereas they examined muscles at a later stage, when regenerative processes such as reinnervation may have begun. Furthermore, our study focused solely on the masseter muscle, while they injected both the masseter and temporalis muscles, potentially triggering different compensatory adaptations that affected fiber type remodeling.

Mitochondrial dysfunction is one of the key factors contributing to muscle atrophy caused by disuse, denervation, and other conditions (Chen et al. 2023). Mitochondrial dysfunction activates catabolic signaling pathways that feedback to the nucleus, driving the expression of muscle atrophy-related genes (Romanello and Sandri 2021). Additionally, the use of the mitochondria-targeted peptide SS-31 was shown to prevent mitochondrial dysfunction and alleviate atrophy caused by muscle inactivity (Min et al. 2011; Powers et al. 2011). Previous studies have reported mitochondrial cristae deformation, swelling, and vacuolar degeneration in the masseter muscle atrophy induced by BTXA injection in both rats and humans (Ma et al. 2018; Moon et al. 2016). In our study, we also observed mitochondrial

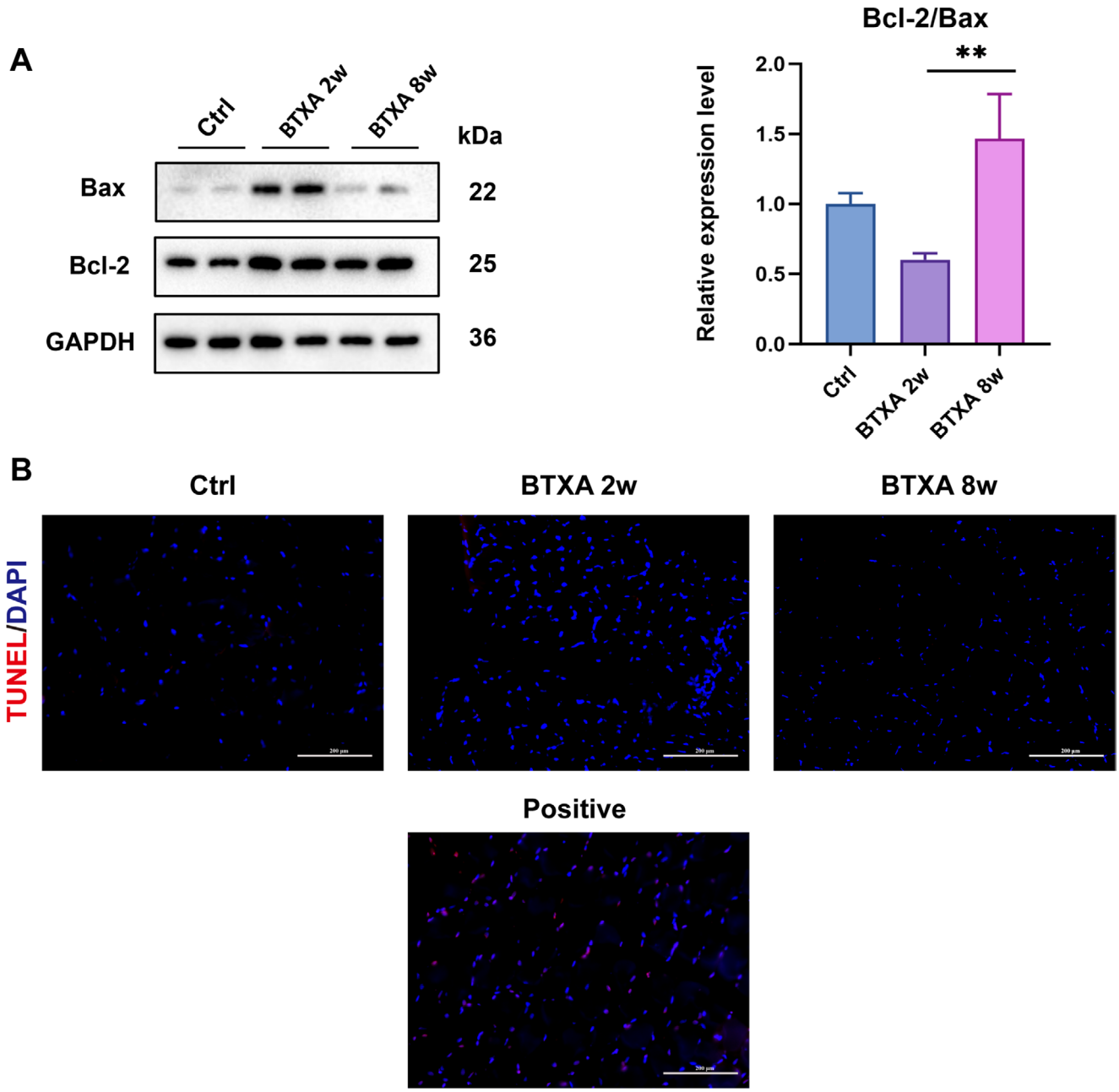

FIGURE 5 | BTXA does not induce cell apoptosis in the masseter muscle. (A) Western blot analysis of BAX and B cl-2 at 2 and 8 weeks post-BTXA injection. (B) TUNEL staining results at 2 and 8 weeks post-BTXA injection. Data are presented as mean ± SEM; $n = 6–8$, $^{**}p < 0.01$ compared with control group.

swelling and cristae disappearance during BTXA-induced muscle atrophy. Moreover, a decrease in ATP content and a reduction in mtDNA copy number were observed. However, by the eighth week post-BTXA injection, atrophied muscle showed partial recovery, accompanied by improvement in mitochondrial morphology, suggesting that mitochondrial dysfunction may play a role in BTXA-induced masseter muscle atrophy.

Mitophagy is a selective degradation process that removes dysfunctional mitochondria to maintain cellular homeostasis, which plays a crucial role in preserving muscle mass (Kubat et al. 2023). On one hand, mitophagy can protect against muscle loss. In chronic models like disuse atrophy (e.g., hindlimb fixation), mitophagy is upregulated as an adaptive response. In this context, inhibiting mitophagy worsens muscle atrophy and mitochondrial dysfunction, confirming its protective role in limiting excessive muscle loss (Rahman et al. 2025). On the other hand, mitophagy can also play a pathological role in atrophy. For instance, the key mitophagy receptor BNIP3 is closely linked to muscle wasting, and in fasting models, RNAi-mediated BNIP3 knockdown attenuates atrophy (Oost et al. 2019). Consistent with this, BNIP3 is upregulated in cancer cachexia, and its knockdown partially rescues muscle mass (Fornelli et al. 2024). Additionally, PINK1/Parkin-mediated mitophagy has been reported to participate in muscle atrophy; in denervation-induced atrophy models, it activated the NFE2L1–proteasome pathway, thereby accelerating muscle protein degradation and atrophy (Furuya et al. 2014). Parkin

deletion in middle-aged mice also spontaneously caused slow-twitch fiber atrophy, MuRF1 upregulation, and Akt signal downregulation (Divino et al. 2025). Therefore, the function of mitophagy is highly dependent on the atrophy stimulus. In our study, we observed an upregulation of autophagy-related molecules LC3II, p62, and Beclin-1 during BTXA-induced masseter muscle atrophy, indicating the activation of autophagy. Transmission electron microscopy further revealed a substantial accumulation of mitochondrial autophagosomes in BTXA-treated muscle, accompanied by a decrease in TOMM20 expression, which further suggests enhanced mitophagy. These findings suggest that BTXA may induce masseter muscle atrophy through the activation of mitophagy. Mitophagy activation is regulated by multiple pathways. The classic PINK1/Parkin pathway is triggered by the loss of mitochondrial membrane potential, where accumulated PINK1 recruits the E3 ubiquitin ligase Parkin to ubiquitinate mitochondrial proteins, guiding selective clearance (Lei et al. 2024). Alternatively, receptor-mediated pathways—independent of Parkin—can also trigger mitophagy. Receptors such as BNIP3, BNIP3L/NIX, and FUNDC1 respond to stressors like hypoxia and bind LC3 or GABARAP via their LIR motifs to recruit autophagosomes for mitochondrial degradation (Lei et al. 2024). Further studies will focus on the signaling pathways that trigger mitophagy in BTXA-induced muscle atrophy.

Furthermore, our study found that apoptosis was not significant during BTXA-induced masseter atrophy. Although previous reports have shown that surgical denervation triggers apoptotic responses in skeletal muscle (Siu and Alway 2005), its mechanism is distinct from the effect of BTXA. Unlike the permanent muscle damage often caused by surgical neurectomy, BTXA-induced changes are typically reversible (Tsai et al. 2010). Our findings are also consistent with a study in the rat detrusor muscle, which reported no evidence of apoptosis following BTXA injection (Watanabe et al. 2010). Mitochondrial dynamics play a critical role in regulating both mitophagy and apoptosis, depending on the type and severity of stress. Mild stress tends to enhance mitochondrial fission to promote mitophagy for quality control (Twig and Shirihai 2011), whereas severe stress can trigger the intrinsic apoptotic pathway (Suen et al. 2008). Both pathways can be activated in muscle atrophy models; for instance, MFN1 deletion drives severe mitochondrial dysfunction and simultaneously activates mitophagy and apoptosis (Yang et al. 2020). Conversely, enhancing mitophagy—for example, via Parkin overexpression—has been shown to suppress apoptosis, oxidative stress, and fibrosis in aging muscle (Leduc-Gaudet et al. 2021). Therefore, our results suggest that BTXA induces masseter atrophy primarily by activating mitophagy.

In summary, this study finds that BTXA can temporarily induce masseter muscle atrophy, with an increase in mitophagy activity potentially being a key factor in this phenomenon. However, the specific mechanisms by which mitophagy contributes to the muscle atrophy induced by BTXA remain unclear. Future experimental studies are needed to elucidate how mitophagy mediates the effects of BTXA on the masseter muscle. Understanding this mechanism could provide new insights into the role of BTXA in clinical applications and its potential side effects.

Author Contributions

Qian-Ying Mao: data curation, formal analysis, writing – original draft. **Zhuo Chen:** formal analysis, validation. **Shang Xie:** methodology. **Ruo-Lan Xiang:** writing – review and editing. **Zhi-Gang Cai:** funding acquisition, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study is available from the corresponding author upon reasonable request.

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