Antihemorrhagic properties of therapeutic botulinum toxin in experimental mice

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Abstract

Botulinum toxin (BoNT) is a potent neurotherapeutic agent that blocks the aberrant release of the neurotransmitter acetylcholine containing vesicles at the neuromuscular junction. While excessive levels of acetylcholine have been linked to inhibition of platelet activities and abnormal bleeding disorders, BoNT treatment can be proposed to regulate the blood coagulation events upon haemorrhagic episodes. Thus, this study examined effect of BoNT treatment on the regulation of biochemical parameters of blood coagulation in experimental aging mice. A set of seven to eight months old experimental mice were intramuscularly injected with therapeutic BoNT (1 U/body Kg weight). After 30 days of treatment, the tail vein transection bleeding assay was performed. Duration of bleeding, clotting time and loss volume of blood were estimated. Further, blood samples were collected from the animals and the blood coagulation parameters like clotting time, light transmission aggregometry and prothrombin time were measured. Result revealed that BoNT injection in experimental animals reduced bleeding time and blood loss during tail vein transection procedure compared to that of control group. The blood samples collected from the BoNT treated mice showed prominent biochemical signatures of platelet aggregation and formation of the fibrin clot compared to that of blood samples from the control animals. Taken together, this study emphasizes that a mild dose of BoNT exhibits antihemorrhagic properties. Thus, therapeutic BoNT can be considered as perspective treatment option against extreme excessive bleeding episodes resulting from traumatic blood vessel injuries and haemorrhagic disease.

Keywords: Botulinum toxin, tail bleeding, platelet aggregation, prothrombin time, coagulation

1. Introduction

Botulinum neurotoxin (BoNT) is potent neurotoxin produced from the Clostridium botulinum bacterium [1]. Very mild purified form of BoNT has been widely implemented as neurotherapeutic agent to block the excessive release of acetylcholine (ACh) from the synaptic vesicles at the neuromuscular junctions [2]. BoNT injections provides effective relief against abnormal muscle stiffness noticed in neuromuscular diseases and movement disorders [2,3]. Besides, BoNT has been widely used in different aesthetic and nonaesthetic facial reconstructive therapies [3,4]. The therapeutic benefits of BoNT has been increasingly recognised against various pathogenic conditions including headache, hyperhidrosis, overactive bladder diseases, eye lid problems, anxiety and dementia [2,3,5,6]. While the therapeutic effects of BoNT from the intramuscular injection site to various distal organs have become increasingly evident, BoNT mediated metabolic, cellular and molecular changes in the blood can be expected for its widespread effects. In the blood, the expression of ACh receptors appear to be prominent in the surface of platelets, while inactivation of platelets resulting from excessive levels of ACh and exposure to its chemical analogues or inhibitors of AChE have been reported be associated with bleeding disorders [7,8]. Considering fact, aberrant levels of ACh noticed in ageing and various pathological conditions may be an underlying cause of progressive deterioration of haemostasis. Therefore, blockade of excessive ACh discharge could be a potential therapeutic strategy for the effective management of haemorrhagic complication in various pathophysiological conditions. Recent reports revealed that intramuscular injection of BoNT enhances the number of circulating platelets in individuals undergoing cosmetic management [9]. Eventually, increased number of platelets has also been reported in the blood sample of ageing mice [6]. It can be

proposed that BoNT mediated changes in the circulation might be associated with regulation of blood coagulation parameters upon haemorrhagic episodes. Therefore, this study has been intended to investigate the haemostatic properties of BoNT at the level of platelet activation and aggregation in association with the changes in key biochemical parameters of blood coagulation in the ageing mice upon experimental bleeding episodes.

2. Materials and methods

2.1 Treatment of BoNT in experimental animals

Seven to eight months old male BALB/c mice (N=12*)* were used to assess the effect of BoNT mediated changes in the biochemical parameters of blood coagulation. The experimental mice were maintained (20–22 °) in the animal house facility, Bharathidasan University at a 12-hr light/dark cycle with free access to feed and water as per the standard procedures. All experiments were conducted in accordance with the approval of the Institutional Animal Ethics Committee (IAEC) (Ref No: BDU/IAEC/P27/2018, 07.08.2018), under the regulation of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

The experimental animals (N=12) were assigned randomly into two groups, namely control group *(*N=6) and BoNT group *(*N=6). BoNT (50U) (Allergan, Dublin, Ireland) was reconstituted and diluted with 0.9% sterile saline. The animals in the test group were intramuscularly injected with 1 unit (U) of BoNT (single dose) per kilogram (Kg) bodyweight (BW) and animals in the control group received the same volume of sterile saline as described in the previous reports [5,6]. After 30 days, animals were subjected

to tail bleeding assay followed by biochemical assessments of platelet activation and prothrombin time in the blood samples as per the standard protocols [10].

2.2 Tail bleeding assay and assessment of volume of blood loss in experimental animals

To investigate the effect of BoNT on the duration of bleeding and amount of blood loss, experimental animals were included for the standard tail bleeding assay. After proper anaesthesia procedure, each animal was placed on a stage and the tail tip was vertically positioned about 2 cm below the body horizon. A distal 10 mm segment of the tail was amputated with the sterile scissors. The tail was immediately immersed in a 15-ml falcon tube containing isotonic 0.9% saline pre-warmed to 37 °C in a water bath (Kemi, India). The duration of bleeding was recorded using a digital stopwatch and each animal was monitored for 30 minutes in order to examine re-bleeding. The difference in the body weight of each animal was measured before and after tail bleeding assay. The volume of blood loss was estimated as volume of blood loss body weight (after tail bleeding) - initial body weight (before tail bleeding) × average blood volume per Kg BW as previously described [11,12]. Next, a drop of blood from each animal was placed on a clean microscopic glass slide and the blood was pulled periodically in an upward direction using a lancet needle at every 30 seconds. The time from collection of the blood to the appearance of the fibrin thread on the glass slide was estimated as the clotting time.

2.3 Estimation of prothrombin time (PT) and Light transmission aggregometry (LTA) in the blood samples

To investigate the effect of BoNT on the prothrombin time, a key parameter of coagulation reaction, each anaesthetized animal, around 250 µl of blood was collected from cardiac puncture and equally distributed in two sterile containers with 3.2% tri sodium citrate buffer (Tulip diagnostics, India) in a 9:1 ratio. One set of blood samples were centrifuged (C-24 Plus, REMI, India) at 2500 g for 15 minutes at room temperature. Further, 100 µl of plasma was separated in a new sterile tube in which 200 µl of prewarmed Uniplastin® sensitive thromboplastin reagent (Tulip Diagnostics, India) was added and incubated in a water bath at 37°C. The time taken for the formation of visible solid gel clot was recorded using a digital stopwatch. The other set of blood samples were centrifuged at 180 g for 10 minutes. The plasma supernatant containing platelets was transferred into another sterile tube and centrifuged again at 1550 g for 10 minutes. As the platelets are sedimented at the bottom of the tube, the upper 2/3rd portion containing platelet-poor plasma (PPP) was carefully removed and added in a separate tube. The platelet pellets were resuspended in remaining 1/3rd lower portion of plasma by gently shaking the tube and considered as platelet rich plasma **(**PRP). These samples were used for the LTA to measure the platelet aggregation as described previously [13]. A 96-well microtiter plate was incubated with 20 µl of phosphate-buffered saline (PBS) in which 25 µl of PRP sample and 5 µl of 2.5 µM adenosine diphosphate (ADP) (SRL, India) were added. In parallel, similar reaction was performed using 25 µl of PPP counterpart. The final assay volume of 50 µl in the microplate was thoroughly mixed for 10 minutes. Absorbance was measured at 595 nm using a microplate reader (iMark™ Bio Rad, India). The degree of platelet aggregation was calculated from the optical density (OD) in the presence of

agonists with reference to PRP and PPP as the percentage of platelet aggregation = (OD of PRP - OD of Sample)/ (OD of PRP - OD of PPP) ×100.

2.4 Statistical analysis

The data values have been represented as mean ± standard deviation (SD). Student t test was applied to measure the statistical significance. All the statistical analysis were made using Graph Pad Prism. The significance level was assumed at $P < 0.05$ unless otherwise indicated.

3. Results

3.1 BoNT injection reduced the duration of bleeding and blood loss in experimental animals with tail tip transection

This study explored the possible role of BoNT treatment on the blood coagulation events in ageing experimental animals. Accordingly, the tail amputation experiment (Fig 1A, 1B) revealed that initial time taken to stop the bleeding (Control = 7.2 ± 1.5 mins vs BoNT = 3.8 ± 1.7 mins) (Fig 1C) and the total duration of bleeding (Control = 9.6 ± 4.3 vs BoNT = 4.8 ± 2.2) (Fig 1D) were significantly reduced in BoNT treated animals than the control group. Due to minimal blood loss resulting from the decreased of bleeding duration the difference between the weight of the body weight before and after tail bleeding assay was found to be highly minimised in BoNT injected animals than the control group (Control = 4.3 ± 1.6 gms vs BoNT =1.7 ± 1.0 gms) (Fig 1E). Eventually, the estimated volume of blood loss was considerably less in the BoNT injected animals compared to animals in the control group (Control = 338 ± 106 µl vs BoNT =75.4 ± 4.0 µl) (Fig 1F).

3.2 Blood derived from BoNT injected animals exhibit reduced clotting time and prothrombin time

The assessment of the clotting time on the microscopic glass slide has been considered as an experimental measure for the degree of intrinsic coagulation pathway (Fig 2A). The time taken for clotting was found to be less in the blood samples of BoNT treated animals than that of the control group. The formation of fibrin threads in the blood sample collected from the BoNT treated animals was also observed to be more rapid than that control animals (Control =157 ± 42 secs vs BoNT = 82 ± 31 secs) (Fig 2B). Further, the outcome of blood clotting time has been validated by the measurement of prothrombin time in the plasma, an important parameter of the extrinsic coagulation pathway. Eventually, the prothrombin time was also significantly reduced in the plasma obtained from the BoNT treated animals than in the plasma of control animals (Control = 17.4 ± 3.0 secs vs BoNT = 10.6 ± 2.2 secs) (Fig 2C).

3.3 BoNT injected animals exhibit enhanced degree of platelet aggregation

While the animals in BoNT treated group showed enhancement in blood clotting events, BoNT mediated effects on the functional characteristic of platelets was subjected for the experimental validation using LTA (Fig 3A,3B), a standard method for evaluating the activation and aggregation of platelets [13]. Notably, the percentage of platelet aggregation was found to be significantly higher in PRP samples obtained from BoNT treated mice than that of the control counterparts (Control = 30 ± 10 % vs BoNT = 60 ± 17 %) (Fig 3C). It indicates that BoNT treatment rendered properties of the platelet adhesion in support of haemostasis.

4. Discussion

The purified form of BoNT has increasingly been recognized as therapeutic agent against various diseases. The present study reveals a putative antihaemorrhagic effect of BoNT against tail transaction bleeding episodes in ageing experimental animals resulting from biochemical changes associated with enhanced haemostasis properties. Existing reports indirectly indicated that intramuscular injection of therapeutic BoNT reduces the risk of bleeding incidences in subjects with different pathological complications [14–16]. Notably, therapeutic BoNT treatment in patients receiving long-term administration of anticoagulants such as warfarin, a vitamin K antagonist (VKA) appears to reduces risk of bleeding problems [14,17]. Yoshida, K., reported that injection of therapeutic BoNT in oromandibular dystonia patients lowered the risk of arterial bleeding [18]. Moreover, therapeutic BoNTs has been recommended as a potent management therapy for spasticity among patients with haemophilia [19]. In general, available antihemorrhagic agents appear to enhance the number and activation of platelets and/or mimic the functions of plasminogen activator inhibitor (PAI), tissue factors (TF) and Von Will Brand factor (VWF), and induction of the transcriptional expression of other clotting factors including factor X and XII [20]. Moreover, drugs with procoagulation properties may also act via the suppression of the expression of tissue plasminogen activator (t-PA) [21]. Abnormal levels of ACh has been reported to interfere with the release of key clotting factors from the kidney into the blood stream [22]. Considering the facts, BoNT mediated enhanced platelet activation and improved coagulation events in ageing experimental animals could directly be related to the decreased levels of ACh in the circulation of the experimental animals, thereby increasing the release of blood clotting factors. Besides, BoNT treatment might also directly be associated with the transcriptional upregulation of clotting factors and compensate the functions of cofactors that are important for the blood

coagulation cascades regardless of ACh. For example, serotonin has been reported to play a crucial role in promoting platelet aggregation and vasoconstriction of surrounding blood vessels [23], while anti-depressant and anxiolytic effects of therapeutic BoNT injection has been reported to act via induced level of serotonin (5-HT) [24]. Therefore, the BoNT mediated effect on blood coagulation might be linked to serotonin. Besides collagen has been identified as the viable thrombogenic component of the sub-endothelium and following vascular damage, circulating platelets appears to be activated and adhered upon the exposure to collagen [25]. While BoNT has been reported to promote the production of collagen, its pro-coagulation effect might involve the collagen mediated platelet activation and adhesion [26].

As prostacyclin, a member of prostaglandin-eicosanoid family of lipid molecules appears to inhibit platelet activation and involve in the remodelling of blood vessel, BoNT has been reported to interfere with synthesis of prostacyclin to reduce inflammatory responses. Transglutaminase (factor XIIIa), stabilize blood clots by cross-linking fibrin strands by catalysing the formation of isopeptide bond between glutamine and lysine residues and therefore stabilize the clot [27]. Previous studies have reported the stimulatory effect of the recombinant light chain of BoNT on transglutaminase activity responsible for the blood clotting event in a dose dependent manner [28]. Choe JE et al., reported the reduced expression of kallikrein in the skin of tissues of BoNT-A treated mice [30]. As kallikrein is a class of serine proteases, enzymes capable of mediating the blood clot lysis and fibrinolysis [29], inhibition of blood clot lysis reactions by BoNT treatment might also be involved in haemostatic properties. Considering the facts, the present study supports the notion that the relatively effective and safe dose of BoNT have putative roles

in blood clotting events through platelet activation, thus it can be considered as an antihemorrhagic medications.

5. Conclusion

Severe trauma, internal haemorrhagic episodes, bleeding disorders, and frequent intake of blood thinner like aspirin and warfarin represents the risk of disabilities and leading cause of death worldwide. Bleeding disorders such as nose gum bleeding, menorrhagia, spontaneous conjunctival haemorrhage and hyphema are associated with defective platelet function. While increased level of ACh namely cholinergic crisis appears to be associated with ageing and many ageing related diseases including diabetes has been known to play a major role in inhibition of platelet activation. Thus, pharmacological blockade of ACh may be a valid therapeutic management for clinical conditions with bleeding complications. Notably, BoNT, an antagonist of ACh release has been used in therapeutic regimen for many human diseases. While conclusive experimental reports have revealed that BoNT is a platelet generating agent. The present study also provides supportive evidence for its role in the platelet activation and blood clotting process in ageing experimental animals. Though the antihaemorrhagic action of BoNT might be directly related to the reduction in the levels of ACh concentration, effects of the BoNT on the expression and activation of the biochemical determinants responsible for the extrinsic and intrinsic clotting factors might not be excluded. In conclusion, BoNT injection in ageing experimental mice mitigates haemorrhagic episodes thus, BoNT mediated molecular and biochemical changes associated with coagulation process could be translated in a therapeutical regime to manage bleeding disorders and excessive blood loss during surgical procedures. As per the FDA guidelines, a mild dose of BoNT has been known to be relatively safe. However, the possible adverse effects associated with

BoNT treatment cannot be completely ignored. Therefore, further studies are required for additional experimental validations of its pro coagulation effects and the further risk assessment needs to be considered.

Authorship contribution

Conceptualization of the study, MK; Planning of experiments, execution of work and data collection, SR; data analysis and interpretation, SR, JMHJ, SS, MK; Initial drafting, SR, MK; reviewing, comments, revisions, SR, JMHJ, SS, MK. All authors have read and agreed to the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

MK has been supported by University Grants Commission-Faculty Recharge Program (UGC-FRP) India. M.K acknowledges a research grant (SERB-EEQ/2016/000639) and an Early Career Research Award (SERB-ECR/2016/000741) from the Science and Engineering Research Board (SERB), government of India M.K and S.S gratefully acknowledges and RUSA2.0, Biological Sciences, BDU for the financial support and UGC-SAP, DST-FIST for the infrastructure of the Department of Animal Science and Department of Bio-Medical Science, Bharathidasan University.

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Figure legends

Fig 1: BoNT treatment reduced bleeding time and loss of blood upon tail vein transection. Representative image of bleeding assay during the tail tip amputation of an experimental mouse (A) The differences in the blood loss from the tail tips cut of a control and BoNT treated mouse in the prewarmed saline containing tubes. The bar graph data represents tail bleeding time (1C), total bleeding duration (1D), and difference in body weight before and after the tail bleeding assay (1E) and estimated volume of blood loss (1F) in control and BoNT treated animals.

Fig 2: Reduced clotting time and prothrombin time in blood samples collected from BoNT treated animals. Representative image for the appearance of the fibrin thread on the glass slide as the estimation of the clotting time (2A) The bar graph data represents clotting time (2B) and Prothrombin time (2C) in the control and BoNT treated animals.

Fig 3: Enhanced platelet aggregation in blood samples of BoNT treated animal

Phase contrast microscopic images of aggregation of platelets obtained from PRP of control (3A) and BoNT (3B) treated animals. The bar graph data represents percentage of platelet aggregation in control and BoNT treated animals (3C).

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