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Novel Use of Botulinum Toxin to Ameliorate Arthrofibrosis: An Experimental Study in Rabbits

HAMID NAMAZI AND SIMIN TORABI

Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

This study aimed to investigate the effects of intra-articular botulinum toxin in preventing arthrofibrosis. Arthrofibrosis was induced in both stifle joints of 20 rabbits by transecting the anterior cruciate ligament under intramuscular anesthesia with ketamine and xylazine. Intra-articular toxin at a dose of 0.6 ml (50 unit) and physiologic saline solution (0.6 ml) were injected into the right and left stifle joints, respectively, 3 times with a 1-week interval between each injection. The rabbits were euthanized in the 12th week via high dose anesthesia to remove the stifle joint. The severity of adhesions was assessed, applying a universal scoring system. Also the stifle joints were histologically evaluated for fibrosis. With regards to severity of adhesion a significant reduction in the adhesion score was observed in the toxin-treated group in comparison to untreated controls with mean ± SE values of 0.2 ± 0.1 and 2.4 ± 0.2, respectively ($p < 0.01$). The histological evaluation showed no significant fibroblast in the toxin-treated group versus dense fibers with mature fibroblasts in the control group. Our results suggest that botulinum toxin demonstrated efficacy in preventing adhesion after knee surgery and all the parameters monitored showed consistent statistically significant improvement.

Keywords. Toxicologic pathology; inflammation; musculoskeletal system; arthrofibrosis; botulinum toxin.

INTRODUCTION

Arthrofibrosis of the knee represents an important concern after knee surgery or trauma. The formation of restrictive adhesions after knee surgery can alter knee biomechanics, leading to stiffness and other severe functional impairments with subsequent pain and cartilage degeneration (Strum et al., 1990; Eakin, 2001). Therefore, a modality which can protect the knee from scar formation is needed.

Although several approaches have been suggested to minimize the knee fibro-adhesive scar formation, however the results of these agents from human clinical trials have been conflicting or involved adverse events (Gomel et al., 1996; Millet et al., 1999; Mentzel et al., 2000; Liew et al., 2001; McCall et al., 2001; Golash et al., 2003; Kim et al., 2004; Brunelli et al., 2005; Wang et al., 2006). In this study, considering the progression of arthrofibrosis from abnormal wound healing problem with the involvement of cellular and biochemical components such as cytokines and growth factors leading to fibrous tissue hyperplasia, the efficacy of botulinum toxin as an anti- adhesive agent in an model in rabbits was investigated.

MATERIALS AND METHODS

Study Group

In the present study 20 mature male New Zealand rabbits weighing 3.2 Kg (2.7–4.5 Kg) were used. The approval of the Ethics committee was obtained. To establish a model of arthrofibrosis, anterior cruciate ligaments of 2 stifle joints were transected (Matthews et al., 2004). Beginning from the first postoperative weeks, botulinum toxin (Botox; Allergan Inc., Irvine CA, USA that 50 units of botox was reconstituted with 0.9% sodium chloride with preservative at a dilution of 0.6 cc per 50 units vial) was injected into stifle joints of right legs of the rabbits (study group) once weekly 0.6 ml (50 unit) for 3 weeks. Physiologic saline solution (0.6 ml) was injected intra-articularly into the stifle joints of the left legs of the rabbits.

Surgical Technique

Preoperatively the rabbits were anesthesized with 10 mg/kg ketamine (i.m.) and 8 mg/kg xylazine (i.m.). Preoperative prophylaxis was done with cephazoline sodium (50 mg/kg i.m.). Skin of stifle joint was shaved, sterilized and draped. Then anterior midline vertical incision was made with medial parapatellar arthrotomy and patella was dislocated laterally. Anterior cruciate ligament was transected with no. 11 scalpels. Normal saline solution was used for intraarticular irrigation.

For the closure of medial retinaculum and skin continuous 4/0 chronic catgut and 4/0 silk sutures were used, respectively. After the procedure, the rabbits were placed in $60 \times 60 \times 40$ cm cages. For postoperative analgesia, 1–2 mg/kg acetaminophen was dissolved in 100 ml drinking water of the rabbits. The rabbits were not immobilized. At 12 weeks postoperatively, the rabbits were sacrificed with high doses (200 mg/kg) of penthotal.

Macroscopic Evaluation

After euthanization of animals, the joints were then exposed by skin parapatellar incision. The presence and severity of osteo-capsular adhesion and secondary synovial synechiae were assessed, applying the following visual scoring system (Rothkopf and Webb, 1991, modified adhesions score): 0, no adhesions; 1, weak, mild, filmy adhesions that can be eliminated by minimal manual traction; 2, moderate adhesions that can be eliminated by manual traction; 3, dense fibrous

Address correspondence to: Hamid Namazi, Department of Orthopaedic Surgery, Shiraz University of Medical Sciences, Chamran Hospital, Shiraz, Iran; e-mail: Namazih@sums.ac.ir

adhesions that must be surgically removed (Rothkopf and Webb, 1991).

Histological Evaluation of Adhesive Tissue

After macroscopic evaluation of adhesions, the stifle joints were excised preserving all connective tissues involved in fibrotic adhesive scar formation. The biopsy was fixed in 10% buffered formalin for 1 week and decalcified for 2 weeks. The tissue was embedded in paraffin and 6 μm transverse sections perpendicular to the femoral axis were stained with hematoxylin-eosin and underwent a blinded microscopic observation by an independent anatomo-pathologist, who received samples without knowing nor treatment group of origin, neither of which was the treatment on study. The following parameters were evaluated: presence of macrophages, fibrosis, presence of granulomatosis, and giant cell reaction.

Statistical Analysis

The statistical analysis was performed using SPSS software. The differences in the number of animals without adhesions in the different treatment groups were evaluated using a Chi-Square test. The effect of botulinum toxin on the reduction of adhesion score in comparison to controls was evaluated by variance analysis. Statistically significant differences were considered when $p < 0.05$. All parameters were also summarized in terms of descriptive statistics (mean and standard error).

RESULTS

The surgery was well tolerated by all animals without any sign of wound infection. But, unfortunately, samples of the 3 rabbits missed and excluded from the study. After the follow-up period a significantly ($p < 0.01$) lower incidence of knees showing adhesion was seen in the toxin-treated with respect to the control group (Fukai et al., 2000, 2% and 100%). With regards to severity of adhesion a significant reduction in the adhesion score was observed in the toxin-treated group in comparison to untreated controls with mean $\pm$ SE values of 0.2 ± 0.1 and 2.4 ± 0.2, respectively ($p < 0.01$) (Table 1).

Macroscopically thicker and more fibrous adhesion, often associated with swelling and microscopic inflammation, developed between the surface of the femoral condyle and the adjacent parapatellar area in the control group. The histological evaluation showed no foreign body reaction in the treated group and only a mild macrophage reaction was evident. Substantial fibrosis was seen in the control group and adhesion appeared as thick and dense fibers with a predominant population of mature fibroblasts. Also, with regard to fibroblast cellularity, a significant reduction was observed in the toxin treated-group in comparison to the control group with mean of 10 cell/HPF and 110 cell/HPF, respectively (Table) (Figures 1 and 2).

TABLE 1.—Adhesion scores in the toxin-treated group and the control group.

	Toxin-treated	Control
1	0	2
2	0	3
3	0	1
4	1	3
5	1	3
6	0	2
7	0	3
8	0	1
9	0	3
10	0	3
11	0	3
12	0	3
13	0	2
14	1	2
15	0	2
16	0	2
17	0	3

0, no adhesions; 1, weak, mild, filmy adhesions that can be eliminated by minimal manual traction; 2, moderate adhesions that can be eliminated by manual traction; 3, dense fibrous adhesions that must be surgically removed.

TABLE 2.—Fibroblast cellularity/HPF (high power field).

	Control group	Toxin treated group
1	110	6
2	112	5
3	113	8
4	111	11
5	108	11
6	109	10
7	109	12
8	110	12
9	106	12
10	108	6
11	112	9
12	114	11
13	110	11
14	110	10
15	119	11
16	118	10
17	114	13

Low fibroblast cellularity in the toxin-treated group versus the control group indicates the efficacy of the botulinum toxin in the prevention of arthrofibrosis.

DISCUSSION

Abnormal proliferation of a fibro-adhesive scar around the connective tissues of a joint can lead to loss of motion, stiffness and pain. Moreover, in the case of joint adhesions, along with articular pain, one of the main problems that can severely affect daily activities is the loss of joint mobility. The presence of scar tissue near the cruciate ligaments and tibial femoral joint is thought to be primarily responsible for loss of extension, with flexion loss resulting from fibrous tissue

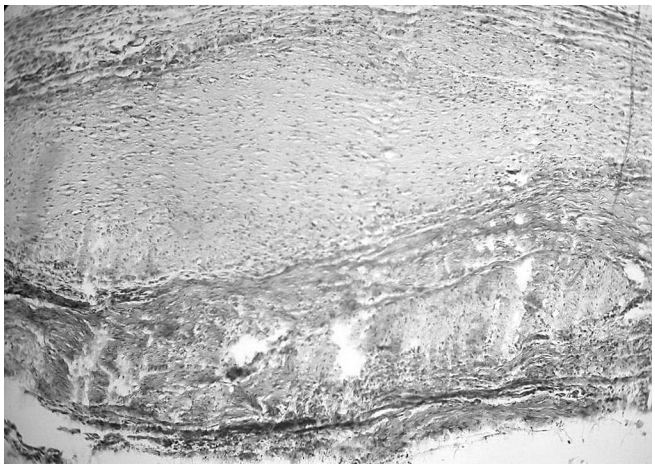

FIGURE 1.—Hypocellular connective tissue shows less fibroblast proliferation.

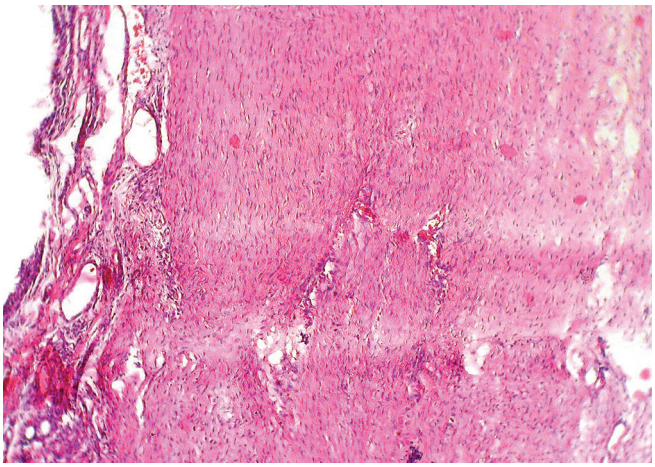

FIGURE 2.—Hypercellular connective tissue shows more fibroblast proliferation.

formation around the patella-femoral articulation and subsequent adherence of the patella to the trochlear groove (Enneking and Horowitz, 1972).

Moreover, several studies were done to solve this problem. Arthroscopic lysis of adhesions has been described as an effective procedure for relief of arthrofibrotic symptoms. Some authors have suggested that this technique is more effective for localized as opposed to diffuse forms of arthrofibrosis, which might regain a satisfactory arc of motion. However, the surgical release or removal of this tissue can stimulate fibrous tissue re-formation with consequent worsening of the symptoms (Cosgarea et al., 1994; Aglietti et al., 1995; Shelbourne et al., 1996). Thus the ideal solution seems to be the using of an anti-adhesion agent to prevent formation of scar tissue. Therefore, to achieve this aim various methods have been attempted such as continuous decorin administration, intra-articular chitosan injection, intra-articular piascledin injection, and local administration of FGF-2 antibodies (Fukui et al., 2001; Xu et al., 2002; Fukui et al., 2001). But, the results of these agents from human clinical trials have been conflicting or involved adverse events (Gomel et al., 1996; Millet et al., 1999; Mentzel et al., 2000; Lieu et al., 2001; McCall et al., 2001; Kim et al., 2004; Brunelli et al., 2005; Wang et al., 2006).

The physiological mechanisms of adhesion formation still remain unclear, however several authors have described the mechanism as an abnormal wound healing process with the involvement of cellular and biochemical components such as cytokines and growth factors leading to fibrous tissue hyperplasia (Paulos et al., 1987; Chang et al., 2000; Fuki et al., 2000). Interleukin-1 is a potent prototypical proinflammatory cytokine implicated in the pathogenesis of arthrofibrosis (Lewthwaite et al., 1995; Blaney et al., 2006). Therefore, as described next, the botulinum toxin minimizes arthrofibrosis by inactivating interleukin 1.

Bacteria produce many enzymes that show extraordinary specificity for mammalian intracellular proteins. The specificity of these bacterial enzymes has not only made them a valuable tool for elucidating the cellular functions of their targets but has also increased our understanding of protein interactions (Holbourn et al., 2005). Clostridium botulinum is no exception, producing 2 classes of enzymes that have very specefic protein targets, neurotoxin A-G and the ADP-ribosyltrans-ferases C2, C3 bot 1, and C3 bot 2 (Holbourn et al., 2005). C2 and C3 bot are part of a larger family of ADP-ribosylating toxins, including diphtheria toxin and cholera toxin, which cleave NAD and transfer ADP-ribose to target proteins.

Although the members of this family have homologous enzymatic domains and similar active sites, these toxins ADP ribosylate and therefore, disable a range of cellular targets (Holbourn et al., 2005). Rho family GTPases controls the assembly of both cell-matrix and cell-cell adhesion complexes. IL-1 receptor signaling complex contained these G proteins, and Rho GTPase is an essential unit for activation of IL-1 inflammatory pathway. C3 transferase exonzyme specifically inhibits Rho GTPase by ADP-ribosylation of amino acid asparagine-41 (Singh et al., 1999; Harmey et al., 2004).

The results of the present study demonstrated that botulinum toxin injection can significantly reduced post-surgery scar tissue formation in the knee. The group of toxin-treated animals developed less clinically relevant adhesions only in 2 cases, while the remaining animals (78%) were completely adhesion-free. One of these was also 1 of the 2 animals in the treatment group that had adhesions.

Macroscopic data were consistent with the result of histology, which revealed very thin adhesion tissue in the same cases, whereas fibrous, dense adhesions were present in all subjects of the control group. Moreover, only 2 out of 7 (29%) control animals had inflammation-free knees, while the remaining 5 had swelling, hematoma, and other inflammatory signs. Histological analysis also confirmed that botulinum toxin did not induce a specific inflammatory reaction and caused a lower fibrotic response compared to the untreated group.

In conclusion, botulinum toxin demonstrated efficacy in preventing adhesion after knee surgery and all the parameters monitored showed consistent statistically significant improvement. In this in vivo study, intra-articular administration showed no unsafe action, confirming the elevated safety profile and biocompatibility of the agent observed in previous clinical trial in different surgical procedures (Brashear, 2005; Crouch, 2006; Goldstein, 2006). Therefore, botulinum toxin may be considered a promising agent to control scar tissue formation after knee surgery such as anterior cruciate ligament reconstruction and meniscus procedure. These promising early experimental results should be confirmed in large, randomized controlled clinical studies.

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