# Effects of masticatory muscle treatment with Botulinum neurotoxin type A on cell division in the mandibular condyle in rabbits

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## 1. INTRODUCTION

The therapeutic and cosmetic roles of botulinum neurotoxins (BTX) have currently expanded to include applications in dentistry. Many patients have received BTX injection in masticatory muscles in an attempt to reduce loading of the temporo-mandibular joint (TMJ) and mandible. This therapy is considered advantageous for alleviating pain in patients with temporo-mandibular disorders (Freund *et al*., 2000; Song *et al.,* 2007), migraine (Binder *et al*., 2000; Silberstein *et al.,* 2000), tension-type headaches (Smuts *et al*., 1999; Blumenfeld *et al*., 2003) and myofascial pain (Ernberg *et al*., 2011; Soares *et al*., 2012; Guarda-Nardini *et al*., 2012). It has also been suggested to be beneficial for osseo-integration after condylar fracture (Canter *et al.,* 2007). However, it is now generally accepted that mechanical loading is essential for the growth, development and maintenance of living tissues. Immobility and unloading could be sufficient to cause bone atrophy.

The TMJ is a synovial, bilateral, ginglymo-diarthrodial joint, and it is formed by the articulation of the condyle of the mandible against the articular fossa and articular eminence of the temporal bone (Wong *et al*., 2006). The mandible with its condyles is the moving bone of the TMJ. The mandibular condyle is an important growth site (Baume *et al*., 1961; van Limborgh *et al.*, 1972) and plays a significant role during mandibular development, remodeling and adapting (Moss and Rankow, 1968; Koski *et al.*, 1968; Meikle *et al.*, 1973; Petrovic *et al*., 1975; Kantomaa *et al.*, 1984). Each condyle is composed of bone covered by a unique cartilage layer exhibiting viscoelastic properties when subjected to tension, compression or shearing forces (Hunziker and Herrmann, 1990). Condylar cartilage also provides regional adaptive growth, endochondral bone growth and a movable articulation. Condylar cartilage is a stiff connective tissue composed of cells in an abundant and highly specialized extracellular matrix. Chondrocyte proliferation and

differentiation are partly regulated by mechanical forces, and cartilage of the mandibular condyle seems to be more sensitive to functional factors than other cartilages (Copray *et al.,* 1986). Consequently, if the TMJ was under unloaded conditions, the joint tissues could show changes.

One way to reduce loading of the TMJ and mandible is to inject masticatory muscles with BTX, because it is a paralytic. A crude measurement of cartilage thickness performed in our lab on rabbits (Matthys, 2012) suggested that paralysis of the masseter using BTX leads to decreases in the cellular portion of articular cartilage in mandibular condyles, although total thickness was unchanged. I hypothesize that this loss of condylar cartilage occurred because BTX therapy inhibited cell division. If this hypothesis is correct, prevention of neuromuscular activity by intramuscular BTX injection would compromise the production, remodeling and accretion of mandibular cartilage.

The purpose of this thesis was to gain a better insight into proliferative activity of cells in the mandibular condylar cartilage in the condition of paralysis of the masseter using BTX-A. A more thorough examination of the cartilage was performed on additional sections from the same specimens that had been examined previously plus additional rabbits treated similarly. The rabbits had been injected with BrdU, a marker of DNA synthesis, before sacrifice, and therefore it was possible to examine the mandibular condylar cartilage in terms of cell division.

The present study was performed to answer the question: “Did BTX-A therapy affect mitotic activity of cells at the mandibular condylar cartilage of the mandibular condyle?”

We expected to see that after injection of BTX-A into one masseter, in the injected-side condylar cartilage, the numbers of immunopositive cells for BrdU, a marker of DNA synthesis, would be degraded after 4 weeks and 12 weeks.

## 2. BACKGROUND

### 2.1. Relation of temporo-mandibular joint and muscles

The temporomandibular joint (TMJ) is the diarthrodial synovial joint of the temporal bone and the mandible separated by a disc. The part of the mandible which mates to the under-surface of the disc is the mandibular condyle and the part of the temporal bone which mates to the upper surface of the disc is the articular eminence and fossa.

The two sides of the TMJ work in unison to perform normal daily functions of the jaw such as speach, food acquisition and mastication (Frank, 1989). Loading in the TMJ may stimulate remodeling, an essential biological response to normal functional demands, ensuring adequate joint mechanics (Stegenga *et al.,* 1989). Muscles are considered to play an important role in the ongoing habitual loading of bone, especially in the masticatory apparatus. Muscle paralysis has been shown to lead to cortical and trabecular bone mass loss (Rafferty *et al.*, 2012; Matthys, 2012).

Several muscle groups insert on the mandible, but the masticatory muscles, due to their strength and functional relation to the jaw bones, may be considered the most important loaders. Although the activity of the other masticatory muscles, the facial muscles, the suprahyoid muscles, as well as additional reaction forces from incisors, molars, and temporomandibular joints also load the mandible, the masseter muscle has been a frequent object in research on mandible -muscle relations (De Jong *et al.*, 2011; John *et al.*, 2011; Cox *et al.*, 2012; Im *et al.*, 2012; Korfage *et al.*, 2012). De Jong *et al*. (2011) reported that masseter activity was a source of low-amplitude, high-frequency bone loading for rabbits and the muscle played an almost continuous role throughout the day. Paralysis of the masseter muscle in growing rabbits and rats resulted in both its own atrophy and in the subsequent growth retardation of the mandible it inserts on (Matic *et al*., 2007; Kim *et al*., 2008).

### 2.2. Mandibular condylar cartilage

In a mandible, condyles are major growth sites and play a significant role during mandibular development. In the mandibular condyle, the subchondral bone, which is mainly formed by endochondral ossification, provides structural support to the overlying articular cartilage. The subchondral bone is composed of cancellous bone and vascular channels. The cartilage and the subchondral bone form a functional entity to withstand the mechanical forces generated during jaw movement and clenching (Jiao *et al*., 2010). The architecture and density of the subchondral bone are deemed to be continuously constructed to accommodate the stress on the fibrocartilage (Giesen *et al*., 2001). Loss of cartilage is often associated with sequestration of the subchondral bone. Changes in subchondral bone may occur prior to the onset of cartilage degeneration (Radin *et al*., 1986). Subchondral bone is an effective shock absorber, and nutrients or cytokines can be transported from the subchondral bone to the overlying cartilage via clefts or channels in the tidemark, so subchondral bone cells influence the overlying cartilage metabolism (Zhang *et al.*, 2012).

The articulating surface of the mandibular condyle is covered by cartilage composed of relatively few cells in an abundant and highly specialized extracellular matrix, which consists mainly of collagens and proteoglycans (Hunziker and Herrmann, 1990). Although cartilage usually contains no nerves, blood vessels or a lymphatic system, glycosaminoglycans (GAG) and water in the matrix permit diffusion of nutrients and metabolic waste products through the matrix.

In growing animals, the cartilage of the mandibular condyle serves not only as an articular cartilage but also as a growth cartilage, similar to the growth plates of long bones (Copray *et al.*, 1988). The mandibular condylar cartilage is composed of chondrocytes at various stages of maturation that according to Ramirez-Yañez *et al*. (2004) are organized into four zones (sub-

layers): (1) superior articular zone (also known as fibrous cell zone); (2) polymorphic (or proliferative) zone; (3) mature zone; and (4) hypertrophic zone.

The articular zone is the outermost surface which receives shearing and frictional loads generated by jaw functions. The cells of this zone appear to be primarily fibroblast-like cells (Appleton, 1975; Mizuno *et al*., 1990). The underlying proliferative zone is mainly cellular with undifferentiated mesenchymal tissue. This highly cellular region not only aids in producing cells for the fibrous zone (Mizuno *et al*., 1990; Copray and Liem, 1989; Silva and Hart, 1967), but also is essentially a cell reservoir for the condylar cartilage, with mesenchymal chondrocyte precursors for the underlying zones (Bibb *et al.*, 1992, 1993; Blackwood, 1966). With the cells being responsible for the adaptation of the matrix content and organization in response to the loading environment, the proliferative zone, despite its relatively small volume, must certainly play an important role in adaptation (Bibb *et al.*, 1993). In the mature and hypertrophic zone, the chondrocytes become hypertrophic and die, and the matrix becomes mineralized. Deeper into this zone, bone and marrow spaces replace the cartilage. This deepest zone is the interface between the condylar cartilage and the subchondral bone.

Growth in the condylar cartilage proceeds from cells exiting the proliferative pool in the polymorphic zone, and then enlarging (Luder *et al*., 1988). The cartilage of the mandibular condyle not only provides endochondral bone growth but also functions as a regional adaptive growth site. The mandibular condylar cartilage is able to remodel its structure in response to mechanical strains (Luder *et al*., 1988; Wadhwa and Kapila, 2008; Ramirez-Yañez *et al*., 2004). The remodeling is achieved by the generation of new chondrocytes and alterations in the composition of the extracellular matrix to achieve a better balance between mechanical stress and the load-bearing capacity of the joint (Shen and Darendeliler, 2005).

Periosteum is a dense fibrous membrane covering the surfaces of bones except for those capped with cartilage. It consists of an outer fibrous layer and an inner cellular layer (cambium). While the mandibular condyle is related to mandibular growth, periosteal activity is responsible for the extensive reshaping of the mandibular ramus (Ochareon and Herring, 2007). Periosteum is not the major focus of this thesis, but because it adjoins the condylar cartilage, adjacent areas were examined.

### 2.3. Effects of mechanical forces

Mature articular cartilage fulfills several functions: it serves to transmit load between skeletal elements, acts as a shock absorber, and provides a practically friction-free gliding surface (Hunziker, 1992). The cartilage of the mandibular condyle is said to be more sensitive to functional factors. The TMJ receives considerable loading during mandibular motion, and this loading contributes to the metabolism of joint tissues. Besides systemic factors such as hormones and local mediators such as growth factors and cytokines, cartilage growth is influenced by mechanical factors. Moreover, local mediators play a role in the translation of mechanical stimuli into cellular responses, and therefore may be involved in the adaptive response of the mandibular condyle to mechanical forces. They are produced locally and are rapidly degraded or inactivated causing them to act only in the immediate environment of the cells that secrete them (Alberts *et al.,* 1989).

### 2.4. Detecting proliferating cells

In order to determine whether the mandibular condyle is undergoing regeneration or degeneration, a way to mark proliferating cells is needed. To identify cells that are in the S phase of replication, originally, scientists used tritiated thymidine, which is a radioactive form of the nucleotide thymidine. However, this method is complicated and time- consuming and involves radiation. Many investigators have found bromodeoxyuridine (BrdU) labeling to be as reliable as

labeling with tritiated thymidine (Lin and Allison, 1993; Grattner, 1982). Monoclonal antibody techniques for detection of BrdU have the advantages of simplicity and speed over standard autoradiography. When cells are synthesizing new copies of genetic material during S phase, they pull nucleotides from the intracellular environment to create a strand of DNA. When BrdU is injected into the bloodstream of a test animal, the chemical becomes available to all cells which are proliferating. Because BrdU can be recognized and stained with an antibody by using immunohistochemical methods, it can be detected by using light microscopy. Any cell that stains positive for BrdU can be said to have undergone DNA replication after the time of injection.

### 2.5. Botulinum neurotoxins and their applications

Botulinum neurotoxins (BTXs) derive from the bacterium *Clostridium botulinum* and include 7 distinct serotypes, identified as A, B, C, D, E, F, and G. Currently, two commercially available subtypes are BTX-A (Botox Cosmetic®, Vistabel®, Vistabex®, Dysport®, Reloxin®, CBTX-A, Prosigne®, Neuronox®, Xeomin®) and BTX-B (Myobloc®, Neurobloc®). When injected intramuscularly at therapeutic doses, the toxins enter the nerve terminals via endocytosis, interact with intracellular proteins (e.g. soluble *N* -ethyl-maleimide sensitive factor attachment protein receptor [SNARE] proteins) and inhibit the vesicular release of the neurotransmitter acetylcholine at the neuromuscular junction (Bhidayasiri and Truong, 2005). BTXs block neuromuscular motor transmission by binding to receptor sites on motor nerve terminals. Receptors for BTXs are also found on autonomic nerve terminals, where they inhibit acetylcholine release at glands and smooth muscle (Meunier *et al*., 2002). Following local injection into muscles, BTXs produce temporary chemodenervation of the muscle, resulting in a localized reduction in muscle activity. Therefore, BTXs have been used as a treatment for a variety of neuromuscular conditions such as dystonias and spasticity, including hypersecretory disorders, oromandibular dystonia,

torticollis, tics, tremor, stuttering, different pain syndromes, detrusor sphincter dyssynergia or overactivity and gastrointestinal smooth muscle/sphincter spasms (Bhidayasiri and Truong, 2005). In many cases, BTX-A is an effective option for the preventive treatment of migraine (Dodick, 2003; Dodick *et al*, 2004; Blumenfeld *et al*., 2004). Due to efficacy of BTXs, they are publicized for aesthetic uses such as the management of facial wrinkles, the correction of asymmetry arising from unilateral muscle paralysis and the treatment of masseteric hypertrophy (Liu *et al.*, 2011). In dentistry, BTXs have been proposed as a treatment for temporo-mandibular disorders (TMD), bruxism, hemifacial spasm, and chronic facial pain. They have also been suggested to be beneficial for osseo-integration after condylar fracture and dental implantology. Botulinum toxin appears relatively safe and effective in clinical practices. Nevertheless, clinical studies with BTX in masticatory muscles have ignored the issue of bone quality. Furthermore, there is still no clear understanding of changes in cartilage and bone in relation to the injected muscle, as well as the biomechanics underlying these.

### 2.6. Effects of BTX employment on cartilage and bone

Injection of BTXs into muscle causes a variety of histological changes, especially in muscle, cartilage and bone. Borodic *et al*. (1994) demonstrated that BTX- A produced a gradient of decrease in average fiber diameter and acetylcholinesterase spread characteristics. Rauch and Hamdy (2006) suggested that intramuscular BTX-A injections could have a deleterious effect on the development of bones that are loaded by the injected muscles. When used experimentally on limb muscles, BTX produces dramatic bone loss in the tibia and distal femur (Manske *et al*., 2010 and 2011; Warner *et al*., 2003). Such osteopenia could be due to unloading or underloading.

The mandible is dominated by the attachments of the large, strong muscles of mastication; therefore, paralysis of the masticatory muscles using BTX also leads to osteoporotic changes in

mandible. Tsai *et al*. (2010) found significant decreases in bone mineral content, cortical thickness, trabecular thickness and ramus height of the mandibular bone on the BTX-A injected side compared with the control side in adult rats. Induction of localized masticatory muscle atrophy with BTX-A also alters craniofacial growth and development. Tsai *et al*. (2011), after injecting BTX-A into temporalis and masseter muscles of growing rats, found that not only were the volumes of the muscles injected with BTX-A smaller, but also the cortical bone thickness and bone mineral density of the skull and mandibular bone structure were reduced. Kim *et al*. (2008) also reported that the mandibles of the developing rats in the BTX-A group had a reduced dimension, compared with the control and saline groups.

Bone loss and reduced cartilage thickness in the craniofacial region, especially in the mandibular condyle, are elicited by underloading after trimming incisors and/or feeding with a soft diet (Chen *et al*., 2009; Hinton and Carlson, 1986; Hinton, 1988; Pirttiniemi *et al*., 2004 among others). Similarly, paralysis of the masticatory muscles using BTX leads to osteoporotic changes in the mandibular condyle, one of the regions of the mandible that receives loading strictly from muscle activity. Kim *et al*. (2008) found that the condylar length and the height of the mandible in the BTX-A developing rats decreased when compared with the control and saline groups. Furthermore, immunoreactions for apoptosis, using the terminal deoxynucleotidyl transferase mediated dUTP nick-end-labeling (TUNEL) method, showed that in specimens of the BTX-A group, a strong positive reaction to TUNEL staining was seen in the proliferative sub-layer of the condyle. Hence, they suggested that BTX-A inhibited mandibular development by causing apoptosis at the proliferation zone of the condylar cartilage in rats.

The specific effects of BTX-A on rabbit condylar bone have been seen in studies from our laboratory (Rafferty *et al*., 2012; Matthys, 2012) using BTX-A injected into one masseter. At 4

weeks after BTX application, the mandibular condyle of the BTX-injected side was underloaded. Bone mass was severely decreased at the injection-side condyle. Twelve weeks after injection, the differential between the injected and uninjected condyles in bone area was still clear with greater trabecular separation and larger marrow spaces on the BTX-A injected side. The present study used this same sample of rabbits 4 and 12 weeks after treatment. Impacts of BTX therapies on DNA replication or DNA repair in tissues have been assessed in only a few studies, all concentrating on muscle rather than the skeleton. While Inagi *et al*. (1998) suggested that BTX might induce a proliferative response in muscle tissue, Chen *et al*. (2002) and Olabici *et al*. (2009) argued that BTX-A did not cause changes in myonuclear production or number and did not appear to promote or improve neogenesis of muscle fibers. Few previous studies have examined the effects of BTX on cell proliferation in the mandible, or its condyle, the major growth site of the mandible. A decrease in proliferation and/or a rising apoptotic rate could account directly for thinning of the condylar cartilage and might also relate to the number of osteoblasts available to contribute to condylar bone.

### 2.7. Statement of the study

Although the fact that BTX blocks the presynaptic release of the acetylcholine and causes paralysis of the masticatory muscles is well known, the possibility of other reactions in diverse tissues affected by BTX injection has not been fully explored. Preliminary examination of our specimens indicated thinning of cellular regions of the articular cartilage on the BTX side (Matthys, 2012). A tendency for the fibrous layer to be thicker (no significance) and the chondrocytic layer to be thinner after BTX treatment was noted. What happened? Did the proliferating cells differentiate into fibroblasts rather than chondrocytes? Was there a reduction in the number of mesenchymal cells proliferating and differentiating into chondroblasts, or a shortened time through the different

stages of chondrocyte maturation and hypertrophy? Alternatively, was there increased cell death, as in the rat study of Kim *et al.* (2008)?

This study was performed to discover effects of BTX-A therapy on mitotic activity of cells at the mandibular condyle via BrdU immunohistochemistry. The sample of BTX-A or saline treated rabbits was derived from the previous experiment (Rafferty *et al*., 2012; Matthys, 2012). Rabbits were used because their TMJs have complex 3-dimensional jaw movements and so they are acceptable models for human mastication (Herring, 2003). Each animal received a unilateral injection of either BTX or saline into one masseter muscle, so the controls were uninjected sides of BTX rabbits and the injected sides of saline rabbits. The hypothesis was that the number of cells labeled with BrdU in condylar cartilage of the BTX side would be fewer than that of the untreated controls at 4 weeks and 12 weeks.

# 3. METHODS AND MATERIALS

## 3.1. Animals and experimental design

Fifty adult five-month-old female New Zealand rabbits had been used in the previous studies. Thirty-one animals received an injection of 10U BTX (Botox ™, Allergan Inc., Irvine, CA) while the other nineteen received an equivalent volume of 0.9% saline (sham animals) into one randomly chosen superficial masseter muscle (at three separate injection points chosen to approximate the location of the motor end plates). Three additional rabbits received no injection. These rabbits also were female and about 5-6 months old at termination. They were considered untreated control animals.

## 3.2. Euthanasia and tissue preparation

Each animal had been anesthetized and injected i.v. with BrdU (40 mg/kg as a 10 mg/ml solution in phosphate-buffered saline (PBS)) seven days prior to sacrifice. With the exception of the untreated controls, the animals were terminated at two endpoints: 4 weeks and 12 weeks, therefore establishing four test groups of rabbits: BTX-injected 4 week, BTX-injected 12 week, saline-injected 4 week and saline-injected 12 week. Each animal had one injection-side condyle and one non-injection side condyle. The 3 untreated controls (average of both condyles) were considered equivalent to the non-injection side of 4 week saline controls. As negative controls for BrdU, 2 additional rabbits had neither masseter nor BrdU injections.

Both condyles of each rabbit had been dissected away from the mandibular ramus, decalcified in Immunocal™ (Decal Chemical Corp., Tallman, NY; formic acid decalcifer), embedded in paraffin, and sectioned coronally (Figure 3.1). From the widest part of each sectioned condyle, 5 slides were chosen; one slide was used as a negative control and four slides were reacted

for BrdU. Slides of hematoxylin and eosin (*H&E)* stained tissue from the previous study (Matthys, 2012) also were used for reference.

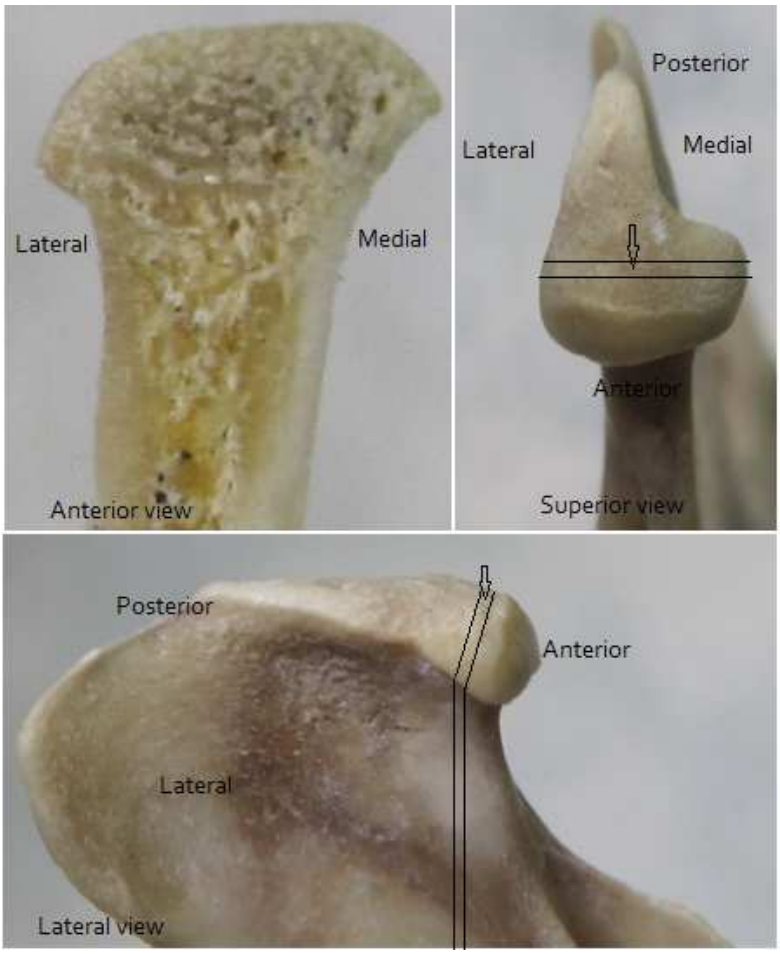


Figure 3.1. Rabbit mandibular condyle in different views. The arrows indicate the area of sections used in the present study.

**BrdU staining**

BrdU immunohistochemical reaction was completed with a BrdU staining kit following the manufacturer's instructions (Becton Dickinson, CA, USA). Slides were deparaffinized with clearene; rehydrated through a graded series of ethanol; incubated in trypsin at $37^0$C for 20 minutes; washed in phosphate buffered saline (PBS); incubated in 2% $H_2O_2$ in $dH_2O$ for 10 minutes; washed in PBS; incubated with a drop of avidin solution and a drop of 1% BSA solution for 15 minutes;

washed in PBS; incubated with a drop of biotin solution and a drop of 1% BSA solution for 15 minutes; washed in PBS; incubated in primary antibody (omitted in negative control), in 1% BSA for 1 hour; washed in PBS; incubated in secondary antibody solution (1drop of biotinylated anti-mouse in 10mls of $dH_2O$) for 30 minutes; washed in PBS; incubated in tertiary antibody solution (streptavidin horseradish peroxidase: 5 ml of $dH_2O$+ 2 drops of reagent A+ 2 drops of reagent B) for 30 minutes; washed in PBS; incubated in DAB solution for 90 seconds; washed in water; counterstained with methyl green; dehydrated and cleared; mounted with Vector Permount and coverslipped.

### 3.3. Immunohistochemical examination

Light microscopic examination and counting of cells positive for BrdU were performed using a Nikon E400 compound microscope. Each section was marked into thirds: medial, central and lateral (Figure 3.2).

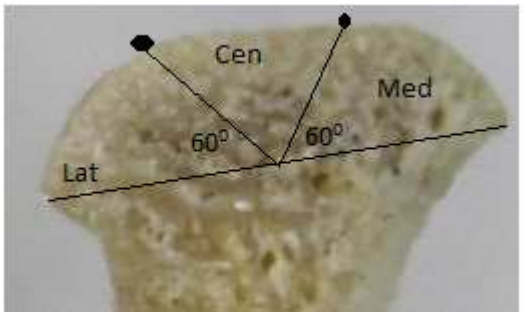


Figure 3.2. Marking for thirds. Each section was divided into thirds (medial (Med), central (Cen) and lateral (Lat)) as shown for quantification of replicating cells in the condylar cartilage.

The superior surface was covered by a layer of dense fibrous connective tissue, designated the “fibrous zone” (Fib Z). Underneath this superior articular zone was a cartilage layer with the chondrocytes arranged in three distinct zones. The proliferative zone (Pro Z) contained small, flat cells underlying and parallel to the fibrous zone. The mature zone was composed of slightly larger ovoid cells. In the hypertrophic zone, the chondrocytes were large spherical cells. We combined

these two zones for counting BrdU labeled cells (M&H Z). The deepest intercellular matrix of this zone was calcified. Further down, where highly hypertrophic chondrocytes were broken down and their surrounding matrices degenerated was the erosive layer. The frontier of the newly formed bone was beneath the erosive layer and was underlain by subchondral bone with marrow spaces. Periosteum was seen on the medial and lateral surfaces of condylar neck. Occasionally, condyles deviated from this general pattern in shape or layering. Subjective notes were made of these findings, with the observer blinded to the identity of the specimens.

Counts of replicating cells in the condylar cartilage were performed in each third in the 3 defined zones: fibrous, proliferative, and mature plus hypertrophic (Figure 3.3). Counts were made directly under the microscope, using the 20x objective. Cells positive for BrdU also were noted in the periostea on the medial and lateral surfaces of the condylar neck, and in the lining cell layers of marrow cavities (Figure 3.3). Cell counting was performed with the observer still blinded to the identity of the specimen. Counts in the same area were averaged from different sections of the same condyle.

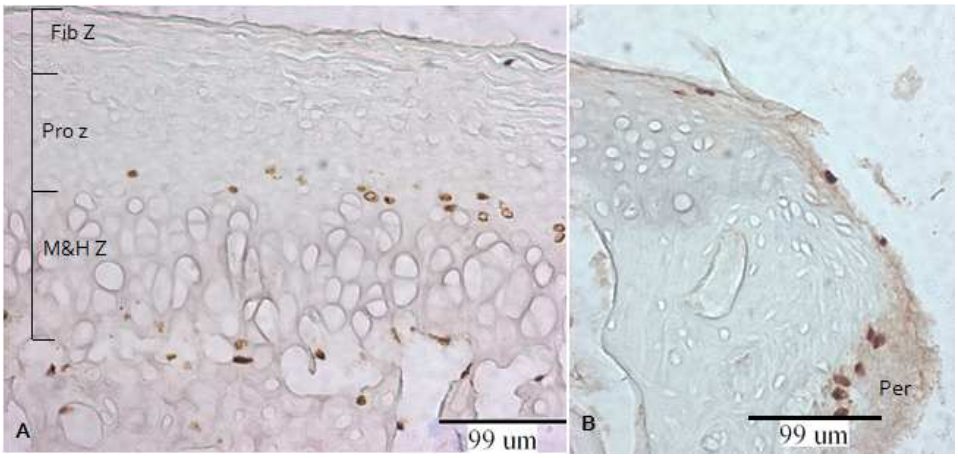


Figure 3.3. A. The zones of the condylar cartilage (Fib Z fibrous, Pro Z proliferative, M&H Z mature plus hypertrophic zones) illustrated in the central third of the left condyle of a BTX 4-week rabbit (# 6832, injected side). B. Labeled cells in the periosteum (Per) near the lateral pole of the condyle of a saline rabbit (uninjected side, 4 weeks, # 9797). BrdU labeled cells were seen in all 3 zones, the periosteum and in the marrow space. Immunohistochemical staining for BrdU and counterstaining with methyl green.

### 3.4. Statistical analysis

#### Measurement error

Measurement error was assessed by counting 25 randomly selected slides several weeks after the first measurement. The method of calculation was that originally described by Dahlberg (1940):

$$S_D = \sqrt{\frac{\sum_{i=1}^{n} d_i^2}{2n}}$$

where $d$ is the difference between the pairs of replicate measurements, $n$ is the number of cases, and $S_D$ is the statistical estimate of the 'true' error (standard deviation).

#### Statistical analysis

Because of the nature of the data (counts), small sample sizes and probable non-normal distribution, non-parametric statistics were used. Condyles from animals terminated 4 weeks after injection and those from animals terminated 12 weeks after injection were analyzed separately. The effects of age on cell division were assessed by comparing 4 week and 12 week samples using Wilcoxon- Mann-Whitney rank sum tests. The effects of BTX treatment on the mitotic activity of cells in the mandibular condylar cartilage were assessed by Wilcoxon signed rank tests between the injected side (BTX) and the contralateral, non-injected side and Wilcoxon- Mann-Whitney rank sum tests between the BTX vs. the saline animals.

# 4. RESULTS

## Missing Data

Some sections were incomplete, presumably because of sectioning error. The tissues within the bone marrow spaces were also often lost during the immunohistochemical procedures, so the numbers of labeled cells seen in the bone marrow are not reliable. A few specimens were lost due to tissue processing problems, diminishing sample size and hence power to detect statistical differences. Table 4.1 summarizes the missing data.

Table 4.1. Missing data

| Group | | | Sample # | Side | Reason |
|---|---|---|---|---|---|
| 4 weeks | BTX | injected side | 4031 | Left | Staining error |
| | | | 4032 | Left | Missing tissue |
| | | | 5222 | Left | Lost |
| | | uninjected side | 4031 | Right | Staining error |
| | | | 4032 | Right | Missing tissue |
| | | | 5222 | Right | Lost |
| | Saline | injected side | 5223 | Left | Missing tissue |
| | | | 9800 | Left | Missing tissue |
| | | uninjected side | 4034 | Right | Lost |
| | | | 9800 | Right | Staining error |
| 12 weeks | BTX | injected side | 4035 | Right | Staining error |
| | | | 6678 | Left | Staining error |
| | | | 6676 | Left | Staining error |
| | | uninjected side | 4035 | Left | Staining error |
| | | | 6676 | Left | Staining error |
| | | | 6678 | Left | Staining error |
| | Saline | uninjected side | 6837 | Right | Missing tissue |

Condyles from the two rabbits that did not receive BrdU injections showed negligible labeling, as did the negative controls (no primary antibody) run with each batch.

### 4.1. Histological morphology

#### 4.1.1. In the animals treated with saline.

In the saline-injected animals, the injected-side and uninjected-side condyles had a similar appearance. In addition, condyles from animals sacrificed 4 weeks after injection looked similar to those sacrificed 12 weeks afer injection. Figure 4.1 shows the typical observed morphological features of a saline uninjected rabbit condyle after reaction for BrdU. The general shape was fungiform. The articular cartilage followed the contour of the condylar head with the thickest portion at the apical region and tapering cartilage thickness towards the medial and lateral poles. BrdU labeled cells, which were identified by the dark brown pigment in their nuclei, were scattered in the proliferative and mature zones. BrdU positive cells of the condylar cartilage were usually more prominent in the central third (Figure 4.2). In the periostea on the medial and lateral surfaces of the condylar neck, BrdU labeled cells were mostly concentrated near the condylar poles (Figure 3.3B). In the lining cells of marrow cavities, BrdU labeled cells were most often seen in the subchondral area. Fewer labeled cells were seen in the bone more distant from the cartilage (Figure 4.3).

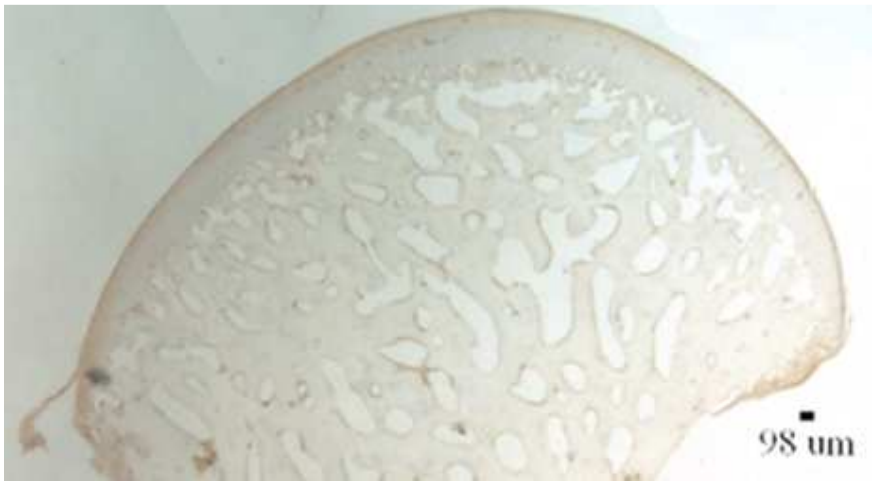


Figure 4.1 Histologic appearance of a saline rabbit condyle (injected side, 4 weeks, # 5221) after BrdU reaction.

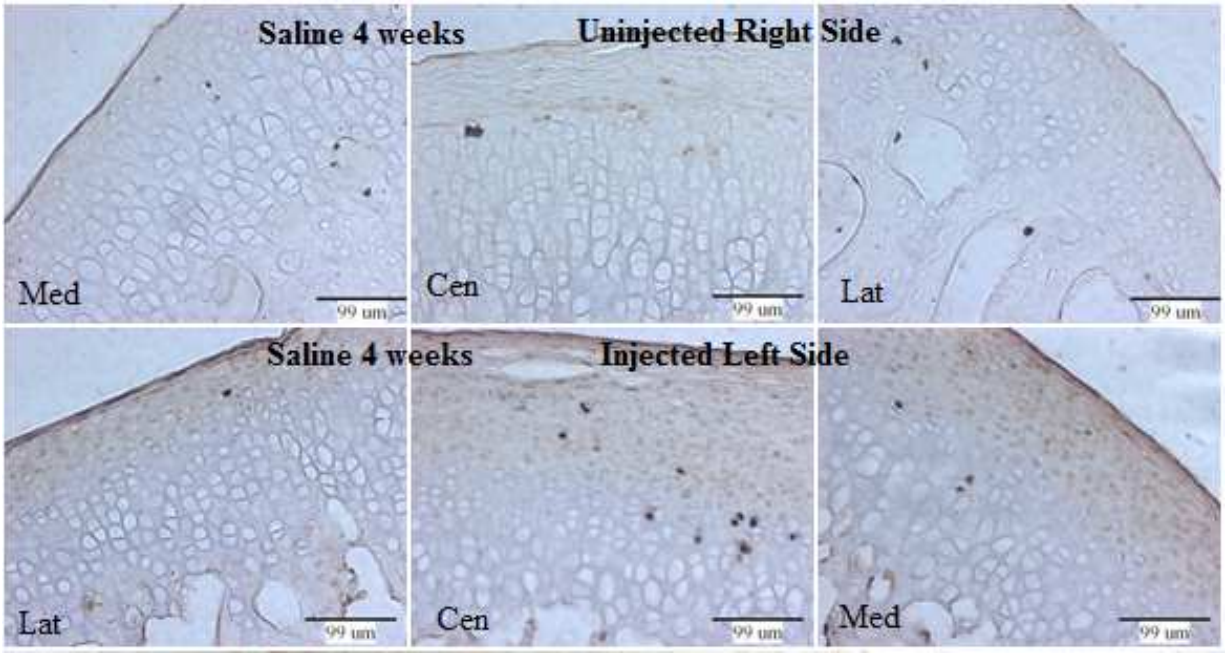


Figure 4.2. Labeled cells in a saline injected animal. BrdU labeled cells are seen in the proliferative and mature zones, especially in the central third of the condylar cartilage. Immunohistochemical staining for BrdU and counterstaining with methyl green, sample # 5221.

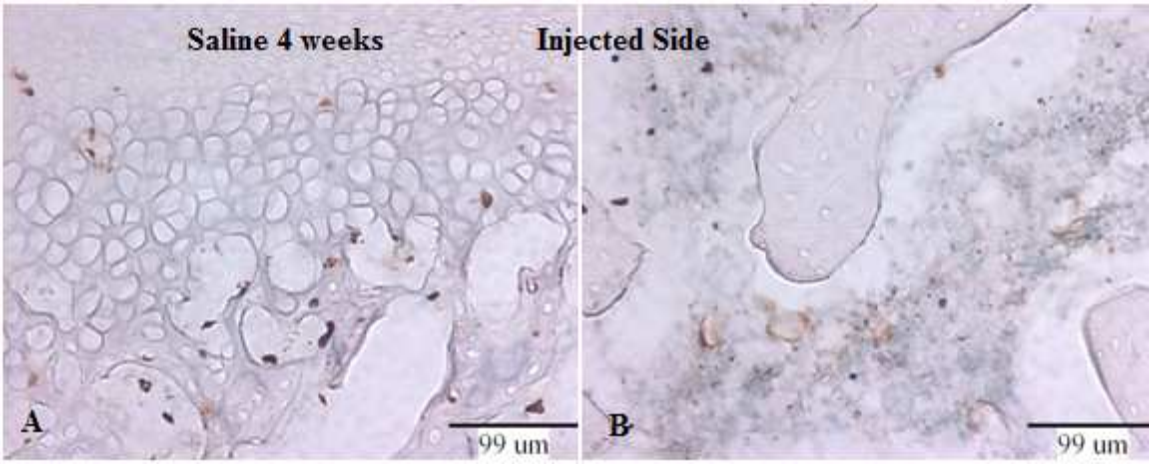


Figure 4.3. Labeled cells in the marrow spaces. In the lining cell layers of marrow cavities, labeled cells were most frequently observed in the subchondral area (A). Fewer labeled cells were seen in the bone more distant from the cartilage (B). Immunohistochemical staining for BrdU and counterstaining with methyl green, sample # 4033.

Two condyles from the saline groups showed a subjective change from typical condylar morphology (Table 4.2). In uninjected sides of # 6831 (4 week group), and # 4038 (12 week group)

both exhibited an irregular shape of the condylar head. This also was seen in one condyle of an untreated rabbit. This condyle, plus both condyles of another untreated rabbits, also exhibited a localized superficial thickening of the cartilage.

### 4.1.2. In the animals treated with BTX

Unusual subjective observations were recorded more frequently for the BTX-treated rabbits, both on the injected and the uninjected side at both timepoints (Table 4.2). In addition to shape irregularities, many condyles displayed local thickening of the fibrous layer, often with disruption of underlying layers (Figure 4.4), usually in the central and medial thirds. This thickened cartilage maintained the shape of the condylar surface by filling bone concavities. Many BrdU positive cells were seen in these areas, including bone marrow spaces as well as cartilage zones (Figure 4.5). A few uninjected-side condyles exhibited islets within the fibrous zone (Table 4.2, Figure 4.6). These were not typically labeled for BrdU.

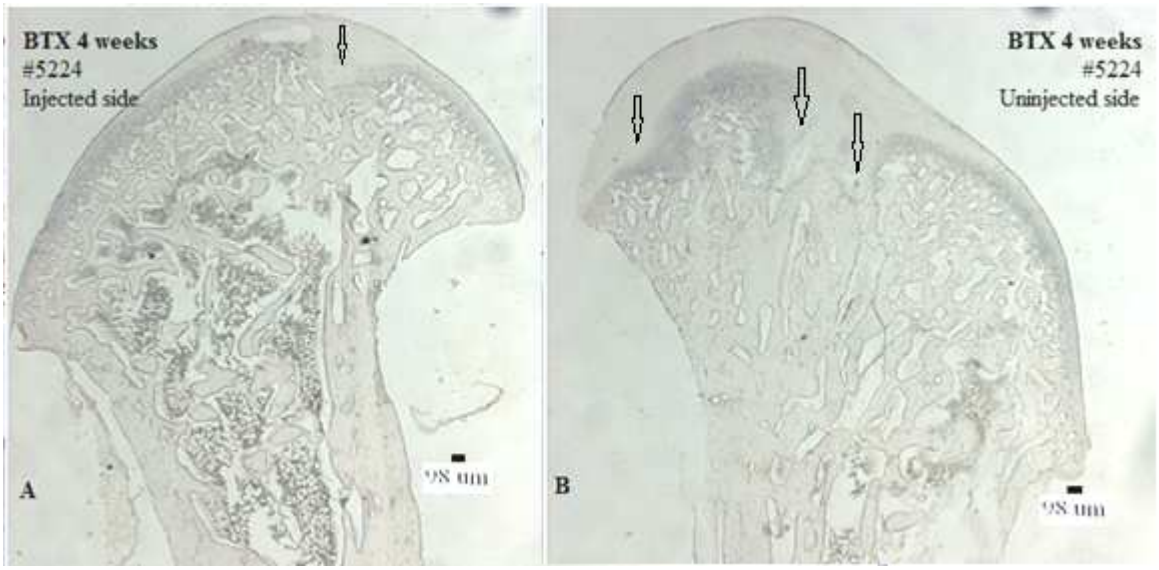


Figure 4.4 Regional thickening of fibrous areas in many condyles of the BTX treated rabbits (arrows). This thickened cartilage maintained the shape of the condylar surface by filling bone concavities. There were extensive invaginations of cartilage into the trabecular bone. The four typical layers of the condylar cartilage often disappeared. Immunohistochemical staining for BrdU and counterstaining with methyl green.

| Table 4.2. Subjective Observations of Condylar Variations | | | | | | |
|---|---|---|---|---|---|---|
| Group | | | Irregular shape | Locally thick fibrous layer | Islets in the fibrous zone | Affected condyles / Total condyles |
| 4 weeks | BTX | Injected side | 9799 R | 5224 L$^{+}$<br>6671 R<br>6674 L$^{+}$<br>6832 R<br>8227 R | | 6/13 |
| | | uninjected side | 5224 R<br>6832 L<br>9798 R<br>9799 L | 5224 R<br>6671 L<br>6672 R$^{+}$<br>6832 L<br>6833 R$^{+}$ | 5224 R<br>6832 L | 7/13 |
| | Saline | injected side | | | | 0/7 |
| | | uninjected side | 6831 L<br>6683 L* | 6683L*$^{+}$<br>6684L*, R*$^{+}$ | | 4/17 |
| 12 weeks | BTX | injected side | | 6836 R$^{+}$<br>9394 L$^{+}$ | | 2/11 |
| | | Uninjected side | 4037 L | 4037 L<br>5219 L<br>6677 L$^{+}$<br>6680L$^{+}$<br>6836 L$^{+}$<br>9793 R<br>9794 R | 4037 L | 7/11 |
| | Saline | injected side | | 6835L$^{+}$ | | 1/9 |
| | | uninjected side | 4038 L | 6835 R$^{+}$ | | 2/9 |

* These rabbits were not treated with any injection and are grouped with saline uninjection sides as additional controls. Both sides contributed to the total of 17.

$^{+}$ These rabbits had unusually high numbers of BrdU-labeled cells, see section 4.3.

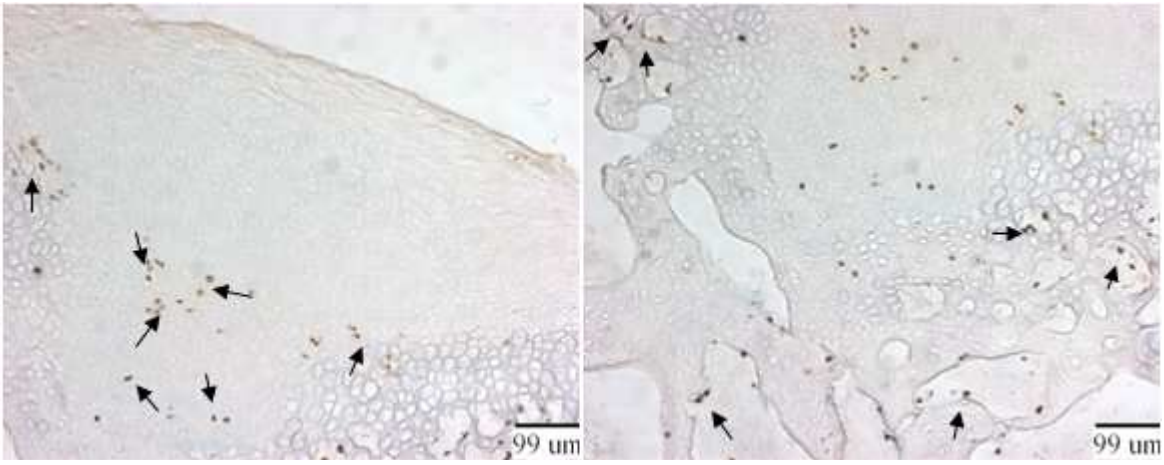


Figure 4.5 Labeled cells in thickened fibrous areas. Many BrdU positive cells (arrows) were seen in the cartilage and bone underlining these thickened areas. Immunohistochemical staining for BrdU and counterstaining with methyl green, injected side, 4 weeks, #8227.

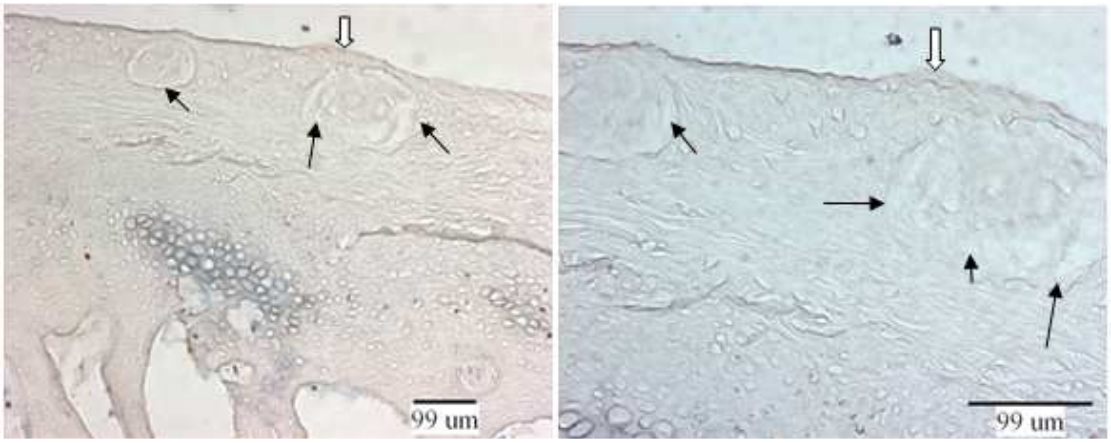


Figure 4.6. Fibrous zone islets (black arrows) sometimes were associated with protuberances (white arrows) on the contour of the condylar head. Immunohistochemical staining for BrdU and counterstaining with methyl green, BTX uninjected side, 12 weeks, #4037.

In some BTX injected side samples (#6672, #8227, #8228, 4 weeks and #5218, #5219, #6677, # 6680, #6836, 12 weeks), many labeled cells were seen near the medial and lateral poles (Figure 4.7). In some sections, regardless of side, it was noted that although the number of BrdU labeled cells in cartilage was very small, in the marrow, the number was rather large (Figure 4.9 A, B). There was often a striking side difference in the number of BrdU labeled cells in the mandibular condylar cartilage (Figure 4.9 C, D), but the injected side sometimes had more, and

sometimes the uninjected side had more. This difference was not observed in the saline groups at either time point, where the two sides were usually similar.

Periosteal labeling was similar to that of saline animals with labeled cells seen in some condyles, mostly concentrated near the condylar poles (Figure 4.8)

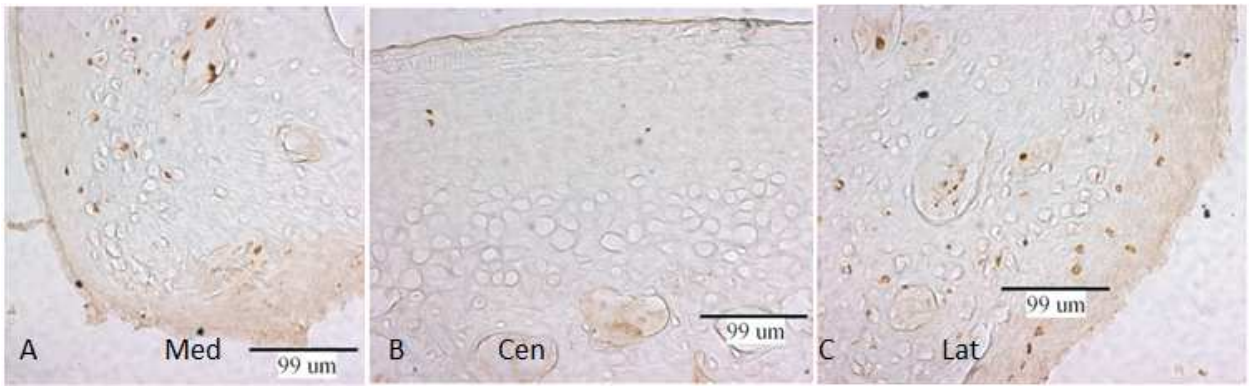


Figure 4.7. Distribution of BrdU labeled cells in the BTX injected-side condyle of a 4 week animals. Labeled cells were concentrated near the medial (A) and lateral (C) poles. Immunohisto-chemical staining for BrdU and counterstaining with methyl green, injected side, 4 weeks, #8228.

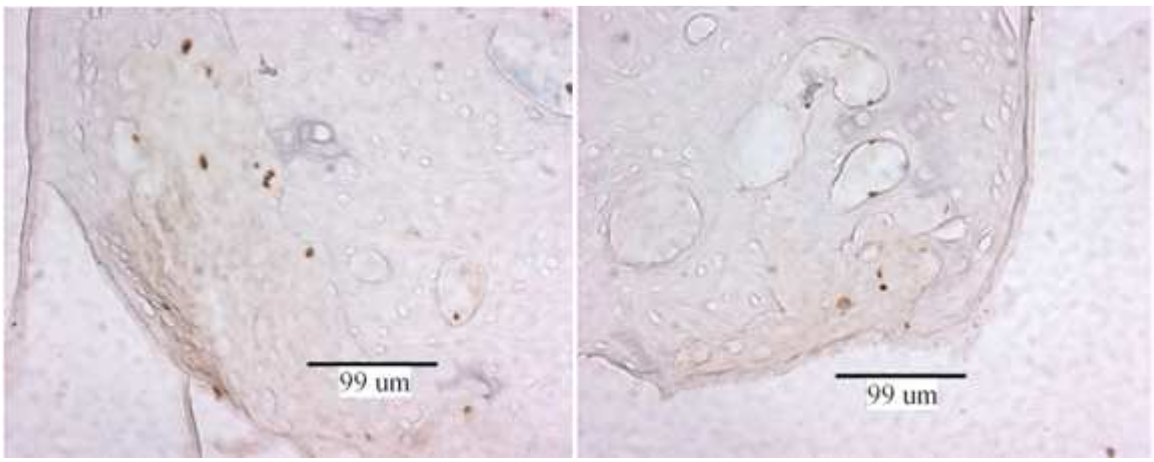


Figure 4.8. In the periosteum, if labeled cells were seen, they concentrated near the medial and lateral poles of the condyle. Immunohistochemical staining for BrdU and counterstaining with methyl green, BTX injected side, 4 weeks, #5224.

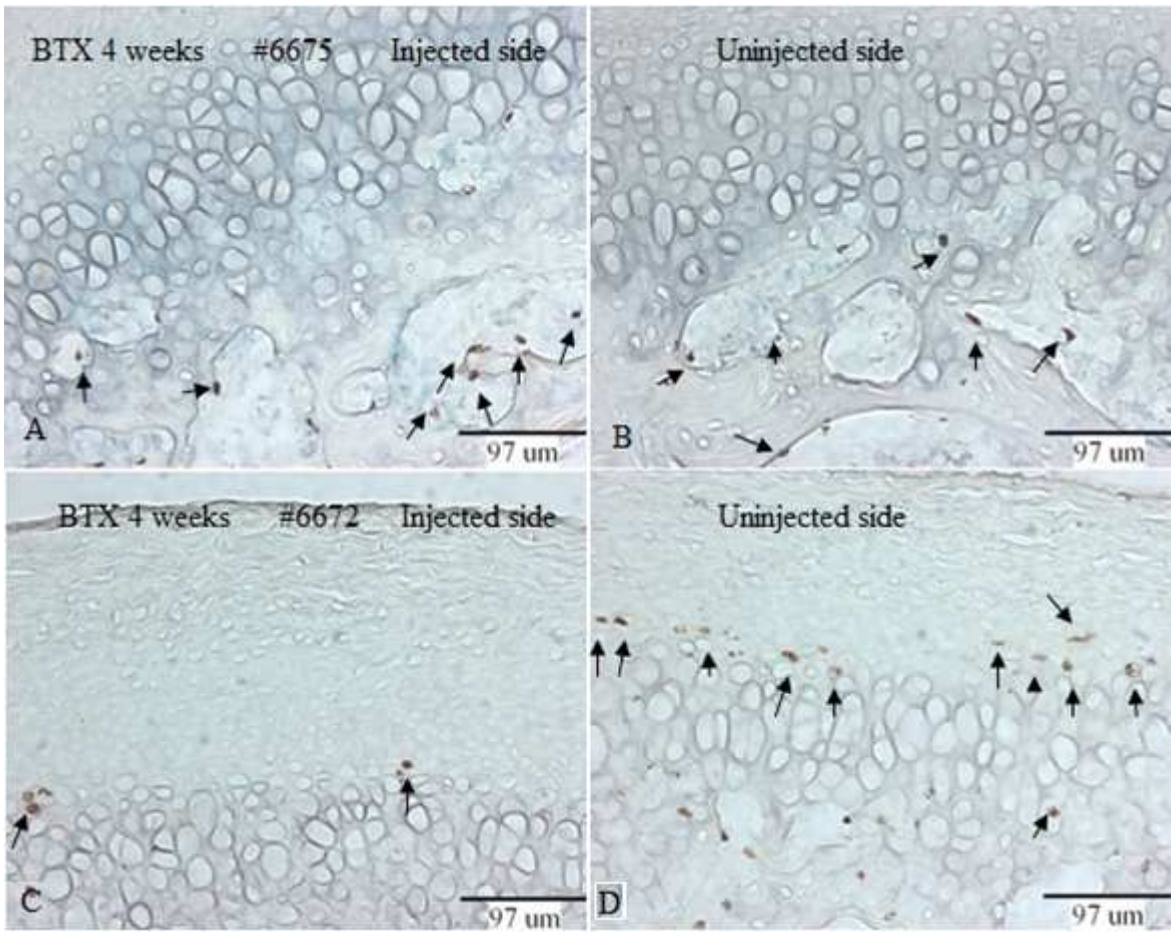


Figure 4.9. A. Difference in the number of BrdU labeled cells. In some sections, BrdU labeled cells (arrows) were scarce in cartilage, but plentiful in marrow spaces (A, B). There was often a difference in the number of BrdU labeled cells in the mandibular condular cartilage between two sides of the same animal treated by BTX (C, D). Immunohistochemical staining for BrdU and counterstaining with methyl green.

### 4.2. Measurement error

Table 4.3 shows the results of the measurement error analysis for cell counts. Except for the fibrous zone, which had very low labeling, repeat counts varied by 5% or less. One cause of error was the difficulty of defining the borders between layers and thirds.

Table 4.3. Error Analysis using Dahlberg's Formula

| | Fib Z | Pro Z | M&H Z | Med | Cen | Lat | Total C |
|---|---|---|---|---|---|---|---|
| Standard Measurement Error | 0.18 | 1.12 | 1.20 | 0.84 | 1.00 | 0.92 | 1.85 |
| Mean of all samples | 1.19 | 24.28 | 22.19 | 13.57 | 18.90 | 15.66 | 48.01 |

### 4.3. Quantification of replicating cells in the condylar cartilage

Using the method of dividing the condyle into thirds (Figure 3.2), the apical portion of the condyles was found either in the central or in the medial third (Figure 4.10).

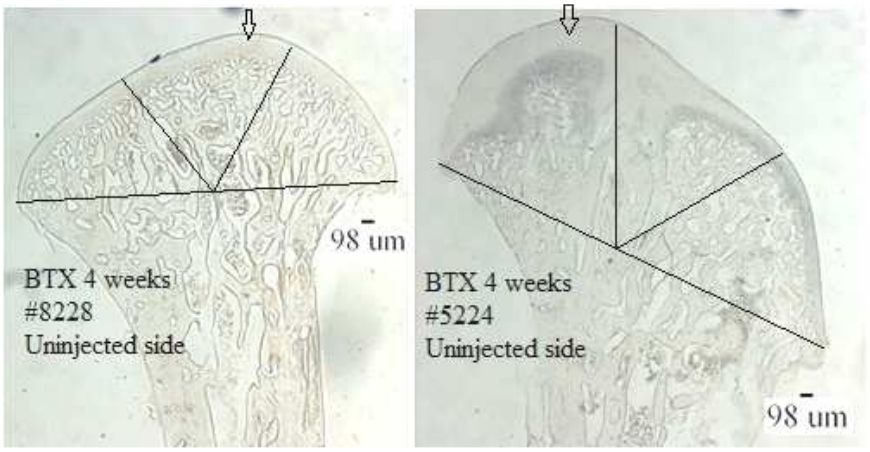


Figure 4.10 Location of the apical portion in either the central (A), or medial third (B).

The following tables (4.4 to 4.11) show the numbers of BrdU labeled cells in mandibular condylar cartilage in all groups by zones - fibrous (Fib Z), proliferative (Pro Z), mature plus hypertrophic (M&H Z) - and by thirds - medial (Med), central (Cent) and lateral (Lat) - , as well as in the total cartilage (Total C). Fractions of a cell result from averaging sections. Total counts over 70 were unusually and are highlighted. Most of these cases had local thickening of the fibrous zone, see Table 4.2.

Table 4.4. BTX injected-side condyles, 4 weeks after injection: counts of BrdU-labeled cells (n=13)

| Sample # | Side | Fib Z | Pro Z | M&H Z | Med | Cen | Lat | Total C |
|---|---|---|---|---|---|---|---|---|
| 5224 | Left | 0.4 | 31.8 | 62.4 | 21.4 | 49.8 | 23.4 | 94.6 |
| 6832 | Right | 0.0 | 9.5 | 12.5 | 7.3 | 8.2 | 6.5 | 22.0 |
| 6633 | Left | 0.3 | 5.8 | 37.5 | 10.5 | 21.8 | 11.3 | 43.6 |
| 6671 | Right | 1.8 | 24.6 | 33.2 | 13.6 | 15.0 | 31.0 | 59.6 |
| 6672 | Left | 0.4 | 16.8 | 11.6 | 12.6 | 9.2 | 7.0 | 28.8 |
| 6673 | Left | 0.0 | 26.5 | 8.0 | 17.0 | 6.5 | 11.0 | 34.5 |
| 6674 | Left | 0.8 | 79.2 | 55.6 | 36.2 | 64.4 | 35.0 | 135.6 |
| 6675 | Right | 0.0 | 11.2 | 7.0 | 3.8 | 9.6 | 4.8 | 18.2 |

| Table 4.4. (continued) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 8225 | Left | 1.0 | 12.4 | 8.8 | 7.6 | 7.4 | 7.2 | 22.2 |
| 8227 | Right | 1.0 | 48.2 | 17.0 | 3.6 | 5.2 | 57.3 | 66.2 |
| 8228 | Right | 2.2 | 46.0 | 54.6 | 40.5 | 17.2 | 45.2 | 102.8 |
| 9798 | Left | 0.4 | 7.3 | 4.6 | 2.7 | 5.9 | 3.7 | 12.3 |
| 9799 | Right | 0.4 | 4.8 | 6.8 | 3.5 | 9.6 | 5.7 | 11.9 |
| Mean | | 0.67 | 24.93 | 24.58 | 13.87 | 17.67 | 19.16 | 50.17 |
| SD | | 0.68 | 21.81 | 21.32 | 12.27 | 18.38 | 17.67 | 39.40 |

| Table 4.5. BTX uninjected-side condyles, 4 weeks after injection: counts of BrdU-labeled cells (n=13) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample # | Side | Fib Z | Pro Z | M&H Z | Med | Cen | Lat | Total C |
| 5224 | Right | 2.3 | 33.3 | 31.0 | 21.3 | 25.5 | 20.0 | 66.8 |
| 6832 | Left | 0.3 | 6.4 | 11.3 | 3.3 | 6.3 | 8.4 | 18.0 |
| 6833 | Right | 0.5 | 48.1 | 57.7 | 12.4 | 54.3 | 39.5 | 106.3 |
| 6671 | Left | 1.5 | 9.3 | 9.0 | 10.8 | 3.8 | 5.3 | 19.8 |
| 6672 | Right | 0.3 | 126.3 | 38.8 | 72.6 | 59.3 | 33.5 | 165.5 |
| 6673 | Right | 0.0 | 6.6 | 5.9 | 9.9 | 2.3 | 0.3 | 12.7 |
| 6674 | Right | 0.0 | 29.0 | 11.3 | 9.8 | 9.3 | 21.3 | 40.3 |
| 6875 | Left | 0.0 | 13.3 | 15.6 | 7.3 | 12.0 | 9.6 | 28.9 |
| 8225 | Right | 0.5 | 18.9 | 15.6 | 4.6 | 20.0 | 10.8 | 35.0 |
| 8227 | Left | 1.5 | 1.1 | 2.3 | 2.3 | 0.8 | 1.9 | 4.9 |
| 8228 | Left | 1.2 | 2.0 | 4.5 | 3.5 | 1.8 | 2.3 | 7.7 |
| 9798 | Right | 0.8 | 2.8 | 2.0 | 2.4 | 1.9 | 1.3 | 5.6 |
| 9799 | Left | 0.6 | 4.6 | 6.6 | 2.4 | 7.2 | 2.2 | 11.8 |
| Mean | | 0.73 | 23.20 | 16.27 | 12.50 | 15.72 | 12.02 | 40.25 |
| SD | | 0.70 | 34.09 | 16.40 | 18.86 | 19.68 | 12.80 | 47.35 |

| Table 4.6. Saline injected-side condyles, 4 weeks after injection: counts of BrdU-labeled cells (n=7) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample # | Side | Fib Z | Pro Z | M&H Z | Med | Cen | Lat | Total C |
| 4033 | Left | 1.0 | 33.4 | 11.3 | 17.6 | 35.2 | 13.6 | 64.0 |
| 4034 | Left | 0.3 | 13.7 | 26.1 | 13.5 | 20.4 | 6.1 | 40.1 |
| 5221 | Left | 0.3 | 17.4 | 33.1 | 19.0 | 25.6 | 6.1 | 50.8 |
| 6831 | Right | 1.4 | 11.9 | 11.3 | 11.6 | 9.1 | 3.75 | 24.5 |
| 8226 | Left | 1.2 | 3.4 | 7.3 | 4.7 | 3.1 | 4.2 | 12.0 |
| 8229 | Right | 2.2 | 11.6 | 19.8 | 3.2 | 19.6 | 10.8 | 33.6 |
| 9797 | Left | 0.0 | 41.5 | 18.9 | 17.3 | 24.1 | 26.5 | 67.9 |
| Mean | | 0.91 | 18.98 | 18.26 | 12.41 | 19.59 | 10.15 | 41.84 |
| SD | | 0.77 | 13.50 | 9.14 | 6.33 | 10.67 | 8.05 | 20.46 |

| Table 4.7. Saline uninjected-side condyles, 4 weeks after injection, counts of BrdU-labeled cells (n=10) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample # | Side | Fib Z | Pro Z | M&H Z | Med | Cen | Lat | Total C |
| 4033 | Right | 2.4 | 29.9 | 37.8 | 18.3 | 33.4 | 18.4 | 70.0 |
| 5221 | Right | 1.8 | 5.5 | 27.2 | 13.2 | 15.7 | 5.7 | 34.5 |
| 5223 | Right | 0.4 | 6.6 | 9.0 | 5.2 | 4.4 | 6.5 | 16.0 |
| 6831 | Left | 1.0 | 15.6 | 10.5 | 7.4 | 5.8 | 14.4 | 27.5 |
| 8226 | Right | 0.5 | 6.0 | 16.3 | 6.9 | 11.8 | 3.9 | 22.5 |
| 8229 | Left | 0.3 | 6.5 | 13.5 | 4.4 | 10.3 | 5.6 | 20.3 |
| 9797 | Right | 1.1 | 9.7 | 45.8 | 10.6 | 39.9 | 6.3 | 56.7 |
| 6885 * | Both | 0.0 | 17 | 13.1 | 11.2 | 14.2 | 4.7 | 30.0 |
| 6684 * | Both | 2.3 | 48.0 | 39.3 | 27.1 | 25.7 | 36.7 | 89.5 |
| 6683 * | Both | 1.1 | 51.9 | 23.5 | 23.4 | 28.9 | 24.3 | 76.5 |
| Mean | | 1.09 | 19.67 | 23.60 | 12.77 | 19.01 | 12.65 | 44.35 |
| SD | | 0.83 | 17.64 | 13.35 | 7.79 | 12.18 | 10.88 | 26.51 |

* These 3 rabbits were not treated with any injection. All were female and about 5 months old. However, #6683 and #6684 both showed subjective alterations of condylar shape (Table 4.2).

| Table 4.8. BTX injected-side condyles, 12 weeks after injection: counts of BrdU-labeled cells (n=11) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample # | Side | Fib Z | Pro Z | M&H Z | Med | Cen | Lat | Total C |
| 4037 | Right | 0.0 | 17.0 | 11.0 | 3.0 | 17.0 | 8.0 | 28.0 |
| 5218 | Right | 0.0 | 25.8 | 5.0 | 14.3 | 9.8 | 6.8 | 30.8 |
| 5219 | Right | 1.3 | 12.2 | 22.2 | 12.0 | 5.2 | 18.5 | 35.7 |
| 6677 | Right | 0.5 | 4.0 | 6.3 | 1.3 | 2.3 | 7.3 | 10.8 |
| 6680 | Right | 0.0 | 4.0 | 37.5 | 20.0 | 15.0 | 6.5 | 41.5 |
| 6836 | Right | 8.6 | 109.9 | 81.1 | 34.3 | 68.9 | 96.4 | 199.6 |
| 6838 | Right | 1.4 | 90.1 | 27.4 | 13.8 | 13.7 | 91.4 | 119.0 |
| 8230 | Right | 0.4 | 3.4 | 10.4 | 1.5.0 | 1.8 | 10.9 | 14.1 |
| 8233 | Left | 0.7 | 3.4 | 9.1 | 3.5 | 6.7 | 3.0 | 13.2 |
| 9793 | Left | 3.0 | 23.7 | 9.7 | 18.2 | 9.5 | 8.7 | 36.3 |
| 9794 | Left | 0.2 | 67.4 | 47.7 | 42.1 | 35.6 | 37.6 | 115.3 |
| Mean | | 1.46 | 32.81 | 24.30 | 21.27 | 16.86 | 26.82 | 58.57 |
| SD | | 2.53 | 38.23 | 23.40 | 19.48 | 19.63 | 34.49 | 60.08 |

| Table 4.9. BTX uninjected-side condyles, 12 weeks after injection: counts of BrdU-labeled cells (n=11) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample # | Side | Fib Z | Pro Z | M&H Z | Med | Cen | Lat | Total C |
| 4037 | Left | 0.4 | 24.6 | 24.8 | 20.0 | 19.4 | 10.4 | 49.9 |
| 5218 | Left | 0.6 | 22.7 | 31.1 | 25.0 | 19.3 | 10.1 | 54.4 |
| 5219 | Left | 0.0 | 10.0 | 11.0 | 15.0 | 1.0 | 5.0 | 21.0 |
| 6677 | Left | 0.5 | 75.5 | 68.0 | 45.5 | 45.0 | 53.5 | 144.0 |

| Table 4.9. (continued) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 6680 | Left | 0.5 | 78.0 | 70.0 | 34.5 | 13.0 | 100.5 | 148.0 |
| 6836 | Left | 8.8 | 55.6 | 44.7 | 35.6 | 31.7 | 41.9 | 109.1 |
| 6838 | Left | 9.4 | 243.0 | 165.8 | 95.5 | 219.5 | 104.0 | 419.0 |
| 8230 | Left | 0.2 | 1.3 | 2.8 | 0.8 | 1.2 | 2.3 | 4.3 |
| 8233 | Right | 3.5 | 16.5 | 45.8 | 20.7 | 13.5 | 31.7 | 65.8 |
| 9793 | Right | 1.4 | 17.6 | 12.4 | 5.8 | 14.4 | 11.1 | 31.4 |
| 9794 | Right | 0.0 | 29.2 | 27.2 | 13.0 | 29.0 | 14.4 | 56.4 |
| Mean | | 2.30 | 52.18 | 45.78 | 28.31 | 37.00 | 34.99 | 100.30 |
| SD | | 3.50 | 68.29 | 45.45 | 25.91 | 61.88 | 36.94 | 115.69 |

| Table 4.10. Saline injected-side condyles, 12 weeks after injection, counts of BrdU-labeled cells (n=9) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample # | Side | Fib Z | Pro Z | M&H Z | Med | Cen | Lat | Total C |
| 4036 | Right | 0.7 | 3.0 | 6.0 | 1.7 | 6.3 | 1.7 | 9.7 |
| 4038 | Right | 1.2 | 17.8 | 19.0 | 8.5 | 14.5 | 15.0 | 38.0 |
| 5217 | Left | 0.2 | 15.2 | 34.0 | 8.4 | 28.2 | 12.8 | 49.4 |
| 5220 | Right | 1.5 | 2.2 | 3.2 | 2.2 | 0.8 | 3.8 | 6.8 |
| 6835 | Left | 8.1 | 121.4 | 98.9 | 61.4 | 103.4 | 63.6 | 228.4 |
| 8231 | Left | 0.4 | 6.6 | 11.1 | 7.8 | 4.6 | 5.8 | 18.1 |
| 8232 | Left | 1.4 | 1.0 | 7.1 | 3.3 | 2.5 | 3.7 | 9.5 |
| 9795 | Right | 0.3 | 12.1 | 35.1 | 14.1 | 17.9 | 15.5 | 47.5 |
| 9796 | Right | 2.9 | 13.0 | 16.7 | 16.1 | 13.0 | 3.4 | 32.6 |
| Mean | | 1.80 | 21.40 | 25.70 | 13.70 | 21.20 | 13.90 | 48.90 |
| SD | | 2.50 | 38.00 | 29.80 | 18.60 | 32.00 | 19.40 | 69.30 |

| Table 4.11. Saline uninjected-side condyles, 12 weeks after injection, counts of BrdU-labeled cells (n=9) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample # | Side | Fib Z | Pro Z | M&H Z | Med | Cen | Lat | Total C |
| 4036 | Left | 0.8 | 1.5 | 8.9 | 3.4 | 2.0 | 5.8 | 11.1 |
| 4038 | Left | 4.0 | 12.0 | 22.4 | 10.8 | 18.8 | 8.8 | 38.4 |
| 5217 | Right | 0.6 | 10.2 | 25.8 | 8.2 | 18.8 | 9.6 | 36.6 |
| 5220 | Left | 1.6 | 34.6 | 40.0 | 25.7 | 22.9 | 27.6 | 76.1 |
| 6835 | Right | 5.7 | 54.7 | 82.9 | 19.1 | 82.3 | 41.9 | 143.3 |
| 8231 | Right | 0.3 | 2.7 | 5.6 | 3.5 | 3.5 | 1.6 | 8.6 |
| 8232 | Right | 0.6 | 8.5 | 10.1 | 10.7 | 3.8 | 4.7 | 19.2 |
| 9795 | Left | 0.9 | 14.0 | 38.0 | 9.1 | 25.0 | 18.7 | 52.9 |
| 9796 | Left | 1.1 | 3.0 | 3.0 | 4.2 | 1.7 | 1.3 | 7.2 |
| Mean | | 1.70 | 15.70 | 26.30 | 10.50 | 19.90 | 13.30 | 43.70 |
| SD | | 1.90 | 17.70 | 25.20 | 7.50 | 25.30 | 13.70 | 43.80 |

Tables 4.12 and 4.13 give counts of labeled cells in the periosteum and marrow spaces. Counts of marrow spaces are less reliable than the cartilage count because of processing problems and therefore were not analyzed statistically.

Tables 4.12. Number of BrdU labeled cells in the periostea on the medial and lateral surfaces of the condylar neck and lining marrow spaces in the BTX treated animals

| | Sample # | Periosteum | | Lining cells | | Periosteum | | Lining cells |
|---|---|---|---|---|---|---|---|---|
| | | Med | Lat | | | Med | Lat | |
| BTX injected side 4 weeks | 5224 | 8.2 | 6.6 | 64.4 | BTX uninjected side 4 weeks | 3.3 | 8.3 | 54.5 |
| | 6832 | 0.5 | 0.8 | 2.3 | | 0.2 | 0.3 | 16.4 |
| | 6833 | 0.2 | 0.2 | 11.8 | | 1.8 | 0.1 | 44.1 |
| | 8225 | 4.8 | 4.2 | 16.8 | | 3.7 | 2.6 | 12.8 |
| | 8227 | 5.7 | 4.7 | 13.7 | | 2.3 | 1.9 | 8.8 |
| | 8228 | 8.6 | 13.2 | 18.8 | | 1.4 | 6.5 | 26.3 |
| | 9798 | 6.5 | 5.6 | 9.1 | | 1.6 | 4.1 | 6.4 |
| | 9799 | 8.0 | 30.6 | 15.7 | | 1.2 | 0.9 | 7.9 |
| Mean | | 5.30 | 8.20 | 19.01 | Mean | 1.91 | 3.06 | 22.1 |
| SD | | 3.33 | 9.87 | 19.03 | SD | 1.06 | 2.98 | 18.11 |
| BTX injected side 12 weeks | 4037 | 10.0 | 2.0 | 10.0 | BTX uninjected side 12 weeks | 3.3 | 0.5 | 36.0 |
| | 5218 | 8.5 | 4.3 | 9.0 | | 3.1 | 4.3 | 21.7 |
| | 5219 | 34.8 | 9.8 | 37.0 | | 6.0 | 8.0 | 32.0 |
| | 6836 | 23.3 | 35.0 | 29.8 | | 7.3 | 7.1 | 23.7 |
| | 6838 | 4.1 | 19.8 | 23.6 | | 15.3 | 3.4 | 24.0 |
| | 8230 | 1.1 | 1.6 | 7.4 | | 2.2 | 1.5 | 4.6 |
| | 8033 | 2.3 | 2.7 | 11.3 | | 5.6 | 10.8 | 28.0 |
| | 9393 | 9.2 | 10.2 | 10.0 | | 9.0 | 3.3 | 18.8 |
| | 9394 | 1.6 | 11.3 | 11.5 | | 3.2 | 16.2 | 16.8 |
| Mean | | 10.55 | 10.73 | 16.62 | Mean | 6.10 | 6.13 | 22.85 |
| SD | | 11.37 | 10.84 | 10.74 | SD | 4.09 | 4.99 | 9.16 |

Table 4.13. Number of BrdU labeled cells in the periostea on the medial and lateral surfaces of the condylar neck and lining marrow spaces in the saline treated animals

| | Sample # | Periosteum | | Lining Cells | | Periosteum | | Lining cells |
|---|---|---|---|---|---|---|---|---|
| | | Med | Lat | | | Med | Lat | |
| Saline injected side 4 weeks | 4033 | 11.8 | 1.0 | 9.7 | Saline uninjected side 4 weeks | 9.1 | 7.9 | 30.1 |
| | 4034 | 0.9 | 0.8 | 8.0 | | 43.6 | 12.2 | 63.8 |
| | 5221 | 11.2 | 37.3 | 59.5 | | 4.8 | 4.7 | 40.7 |
| | 6831 | 0.9 | 2.4 | 22.9 | | 1.1 | 3.8 | 18.0 |
| | 8226 | 5.8 | 8.9 | 10.8 | | 5.5 | 8.3 | 26.6 |
| | 8229 | 27.0 | 8.3 | 18.4 | | 2.9 | 4.4 | 8.3 |
| | 9797 | 5.6 | 10.8 | No data | | 6.0 | 7.3 | 17.8 |

| Table 4.13. (continued) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Mean | | 9.01 | 9.90 | 21.51 | Mean | 10.42 | 6.91 | 29.3 |
| SD | | 9.04 | 12.75 | 19.46 | SD | 14.84 | 2.94 | 18.4 |
| Saline injected side<br><br>12 weeks | 4036<br>4038<br>5217<br>5220<br>8231<br>8232<br>9795<br>9796 | 2.4<br>2.8<br>20.2<br>3.0<br>1.8<br>5.9<br>7.0<br>1.6 | 0.8<br>1.6<br>24.8<br>3.0<br>3.8<br>2.2<br>14.2<br>2.0 | 6.0<br>12.0<br>35.0<br>10.8<br>12.2<br>5.8<br>26.8<br>3.7 | Saline uninjected side<br><br>12 weeks | 3.0<br>15.6<br>7.5<br>1.8<br>2.2<br>1.4<br>9.4<br>2.1 | 5.1<br>8.4<br>10.0<br>3.6<br>1.1<br>2.5<br>11.3<br>2.3 | 26.7<br>22.0<br>39.7<br>42.0<br>9.5<br>5.3<br>6.0<br>2.9 |
| Mean | | 5.59 | 6.6 | 14.04 | Mean | 5.38 | 5.53 | 19.25 |
| SD | | 6.21 | 8.53 | 11.08 | SD | 5.07 | 3.86 | 15.73 |

### 4.4. Distribution of BrdU positive cells in the condylar cartilage of the animals treated with saline.

Table 4.14 and Figure 4.13 summarize the quantitative analysis of BrdU-labeling in different zones of the condylar cartilage in the saline control animals. Labeling in the fibrous zone was consistently much lower than in the proliferative or mature-hypertrophic zones. There was an inconsistent difference between the proliferative and the mature-hypertrophic zones, probably reflecting the difficulty in distinguishing the border between them.

| Table 4.14. Distribution of BrdU positive cells in the 3 zones of the condylar cartilage in the saline treated animals (mean[standard deviation]) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Saline | Sides | Fib Z | Pro Z | M&H Z | Friedman test | Wilcoxon signed rank tests | | |
| | | | | | | Fib Z vs Pro Z | Fib Z vs M&H Z | Pro Z vs M&H Z |
| 4 weeks | Injected side n=7 | 0.91 [0.77] | 18.98 [13.50] | 18.26 [9.14] | 0.005 | 0.018 | 0.018 | 1.000 |
| | Uninjected side n=10 | 1.09 [0.83] | 19.67 [8.25] | 23.60 [13.35] | 0.001 | 0.005 | 0.005 | 0.005 |
| 12 weeks | Injected n=9 | 1.80 [2.50] | 21.40 [38.00] | 25.70 29.80] | 0.001 | 0.011 | 0.008 | 0.086 |
| | Uninjected n=9 | 1.70 [1.90] | 15.70 [17.70] | 26.30 [25.20] | 0.000 | 0.008 | 0.008 | 0.012 |

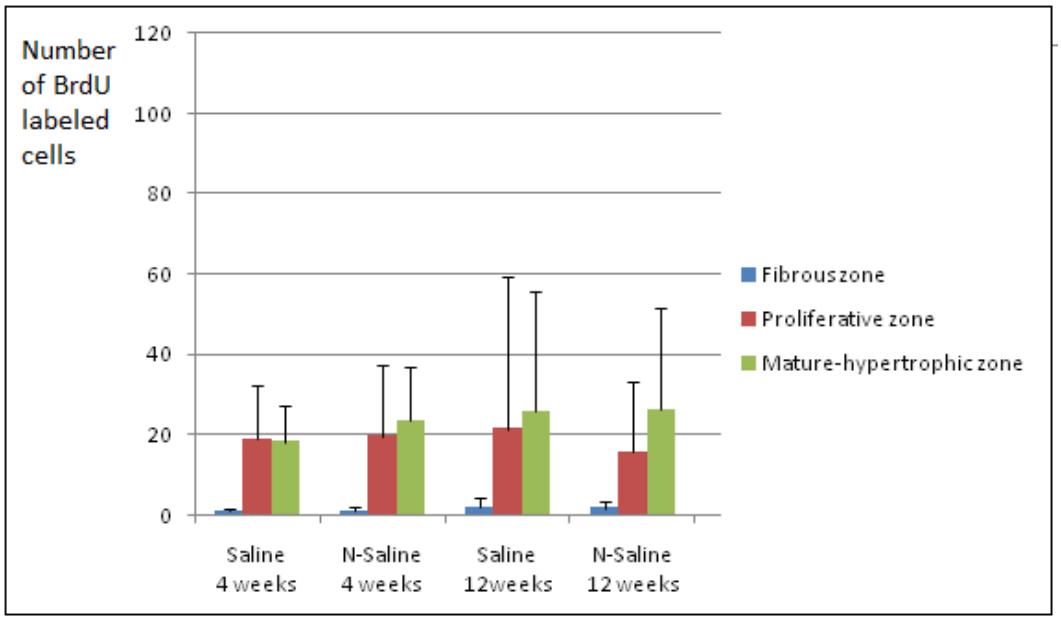


Figure 4.11. BrdU positive cells in the fibrous, proliferative and mature-hypertrophic zones of the condylar cartilage in control rabbits. BrdU labeled cells were more numerous in the proliferative and mature plus hypertrophic zones than in the fibrous layer at both timepoints both on the side treated with saline (Saline) and the uninjected side (N-Saline and untreated). Mean, standard deviation.

Table 4.15 and Figure 4.12 combine cartilage zones in order to compare medial, central and lateral thirds. A trend of more labeled cells in the central third than the other regions was seen in both sides of the saline treated samples, but it was not statistically significant.

Table 4.15 Distribution of BrdU positive cells in medial (Med), central (Cen) and lateral (Lat) thirds of the condylar cartilage in the saline treated animals (mean[standard deviation])

| Saline | Sides | Med | Cen | Lat | Friedman test |
|---|---|---|---|---|---|
| 4 weeks | Injected n=7 | 12.41 [6.33] | 19.59 [10.67] | 10.15 [8.05] | 0.097 |
| | Uninjected n=10 | 12.77 [7.79] | 19.01 [12.18] | 12.65 [10.88] | 0.273 |
| 12 weeks | Injected n=9 | 13.70 [18.50] | 21.20 [32.00] | 13.90 [19.40] | 0.581 |
| | Uninjected n=9 | 10.50 [7.50] | 19.90 [25.30] | 13.30 [13.70] | 0.919 |

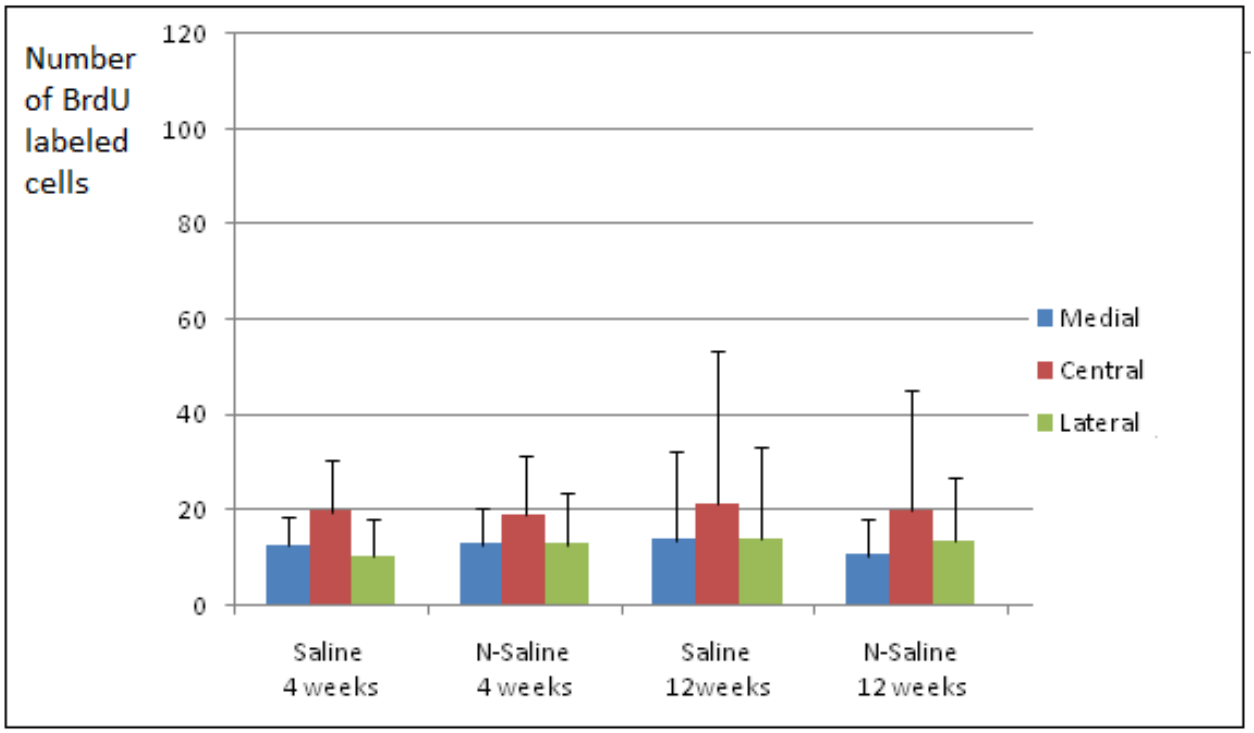


Figure 4.12 BrdU positive cells in the medial, central and lateral thirds of the condylar cartilage in saline treated control animals. Although there were more labeled cells in the central third than the other regions on both sides, the differences were not statistically significant. (Mean, standard deviation)

Although injection of saline into one masseter had no apparent qualitative or quantitative effect on the condyle of that side, for purposes of comparison with the BTX-treated rabbits, only the uninjected sides plus the untreated rabbits (sides averaged) were used.

### 4.5. Distribution of BrdU positive cells in condylar cartilage of the animals treated with BTX.

As shown in table 4.16 and Figure 4.13, the zonal distribution of labeled cells was similar to that of saline controls in that labeling of the fibrous zone was much lower than that of the proliferative and mature- hypertrophic zones with no clear difference between the latter two zones. Variability was very high, especially on the uninjected side and in the 12 week animals.

| Table 4.16 Distribution of BrdU positive cells in the 3 zones of the condylar cartilage in the BTX treated animals (Mean [Standard deviation]) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| BTX | Sides | Fib Z | Pro Z | M&H Z | Friedman test | Wilcoxon signed rank tests | | |
| | | | | | | Fib Z vs Pro Z | Fib Z vs M&H Z | Pro Z vs M&H Z |
| 4 weeks | Injected side n=13 | 0.67 [0.68] | 24.93 [21.81] | 24.58 [21.32] | 0.000 | 0.001 | 0.001 | 0.861 |
| | Uninjected Side n=13 | 0.73 [0.70] | 23.20 [34.09] | 16.27 [16.40] | 0.000 | 0.002 | 0.001 | 0.944 |
| 12 weeks | Injected side n=11 | 1.46 [2.53] | 32.81 [38.23] | 24.30 [23.40] | 0.001 | 0.003 | 0.003 | 0.328 |
| | Uninjected Side n=11 | 2.30 [3.50] | 52.18 [68.29] | 45.78 [45.45] | 0.000 | 0.003 | 0.003 | 0.424 |

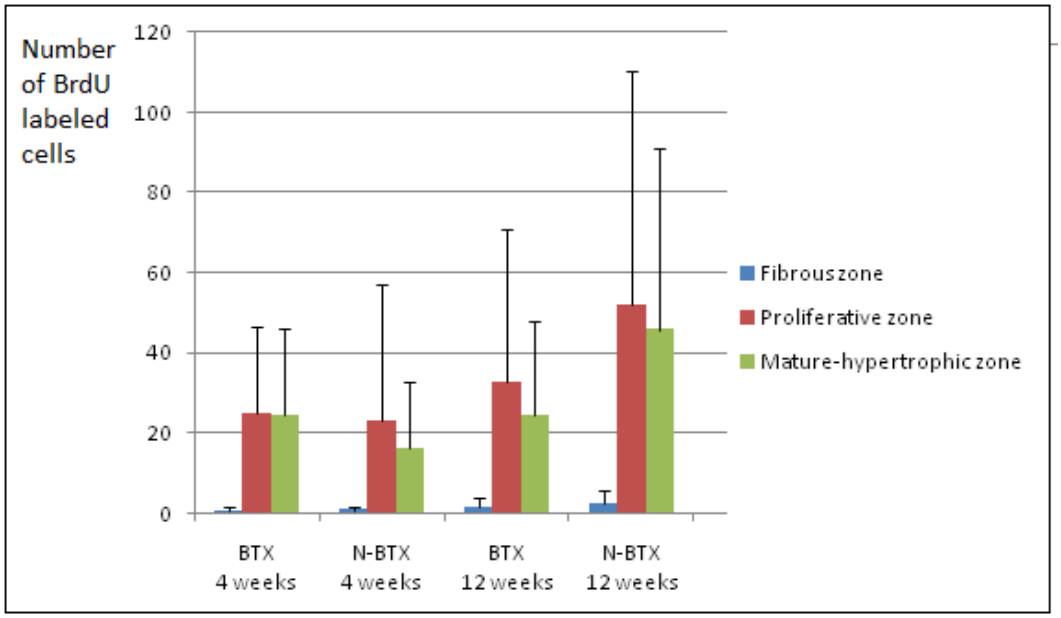


Figure 4.13 BrdU positive cells in the fibrous, proliferative and mature-hypertrophic zones of the condylar cartilage of the BTX treated rabbits. BrdU labeled cells were more numerous in the proliferative and mature plus hypertrophic zones than in the fibrous layer at both timepoints and on both sides. (Mean, standard deviation)

Table 4.17 and Figure 4.14 combine zones to compare medial, central and lateral thirds of the condylar cartilage. No significant differences were found, but at both timepoints, the injected side had the most labeled cells in the lateral third, whereas the uninjected side had the most centrally.

Table 4.17 Distribution of BrdU positive cells in medial (Med), central (Cen) and lateral (Lat) thirds of the condylar cartilage in the BTX treated animals (Mean [Standard deviation])

| BTX | Sides | Med | Cen | Lat | Friedman test |
|---|---|---|---|---|---|
| 4 weeks | Injected side n=13 | 13.87 [12.27] | 17.67 [18.38] | 19.16 [17.67] | 0.199 |
| | Uninjected side n=13 | 12.50 [18.86] | 15.72 [19.68] | 12.02 [12.80] | 0.584 |
| 12 weeks | Injected side n=11 | 21.27 [19.48] | 16.86 [19.63] | 26.82 [34.49] | 0.913 |
| | Uninjected side n=11 | 28.31 [25.91] | 37.00 [61.88] | 34.99 [36.94] | 0.529 |

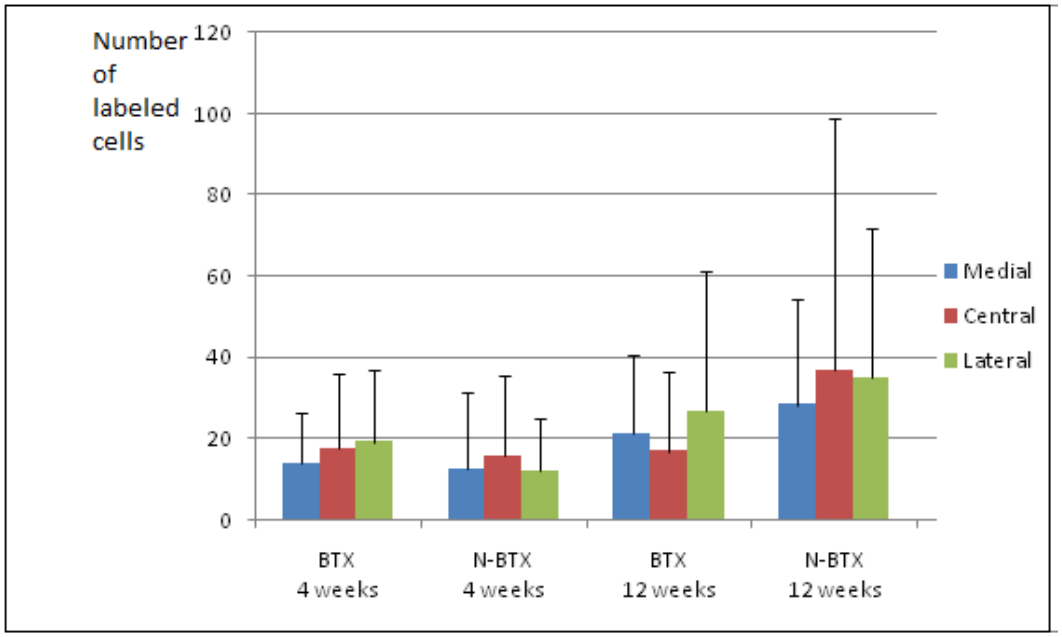


Figure 4.14 BrdU positive cells in the medial, central and lateral thirds of the condylar cartilage in the BTX treated animals. There was no significant difference in labeling in thirds. (Mean, standard deviation)

### 4.6. Does mitotic activity of cells at the mandibular condylar cartilage change with age?

Table 4.18 compares the number of labeled cells in the total condylar cartilage at 4 weeks with 12 weeks to determine if age affected mitotic activity of cells in condylar cartilage. Totals were nearly identical in the saline animals, indicating that age did not affect mitotic activity of cells in 6.5 (the 4 week group) vs 8.5 month (the 12 week group) old rabbits (Wilcoxon- Mann-Whitney rank sum test). However, a similar set of tests for the BTX treated animals, showed a tendency for mitotic activity to increase with time on the non-injected side (P=0.063). This is presumably a treatment effect rather than an age effect, because it occurred only in BTX-treated animals.

Table 4.18 Timepoint changes in mitotic activity of cells in the mandibular condylar cartilage 4 weeks vs 12 weeks after injection (mean [standard deviation])

| | side | 4 weeks | 12 weeks | Wilcoxon- Mann-Whitney rank sum test |
|---|---|---|---|---|
| Saline | Injected | 41.84 [20.46] | 48.90 [69.30] | 0.336 |
| | Uninjected | 44.35 [26.51] | 44.18 [43.40] | 0.368 |
| BTX | Injected | 50.17 [39.40] | 58.57 [60.08] | 0.832 |
| | Uninjected | 40.25 [47.35] | 100.30 [115.69] | 0.063 |

### 4.7. Does BTX-A therapy of the masseter affect mitotic activity of cells at the condylar cartilage?

The effect of masseteric injection of BTX on cell labeling in the condylar cartilage was tested first by paired comparisons between the uninjected and injected sides using Wilcoxon signed rank test. The paired data indicate no significant differences in mitotic activity between two sides in any zone or third (Table 4.19). It is notable, though, that at 4 weeks every comparison of averages (except for the essentially unlabeled fibrous zone) showed more labeled cells on the BTX-injected

side, whereas at 12 weeks every comparison of averages showed more labeled cells on the uninjected side. Variation was high.

Table 4.19. Injected vs uninjected sides of BTX-treated animals: mitotic activity of cells the mandibular condylar cartilage (mean [standard deviation])

| | Side | Fib Z | Pro Z | M&H Z | Med | Cen | Lat | Total C |
|---|---|---|---|---|---|---|---|---|
| 4 weeks | BTX injected | 0.67 [0.68] | 24.93 [21.81] | 24.58 [21.32] | 13.87 [12.27] | 17.67 [18.38] | 19.16 [17.67] | 50.17 [39.40] |
| | BTX un-injected | 0.73 [0.70] | 23.20 [34.09] | 16.27 [16.40] | 12.50 [18.86] | 15.72 [19.68] | 12.02 [12.80] | 40.25 [47.35] |
| Wilcoxon signed rank test | | 0.929 | 0.382 | 0.249 | 0.173 | 0.402 | 0.382 | 0.345 |
| 12 weeks | BTX injected | 1.46 [2.53] | 32.81 [38.23] | 24.30 [23.40] | 21.27 [19.48] | 16.86 [19.63] | 26.82 [34.49] | 58.57 [60.08] |
| | BTX un-injected | 2.30 [3.50] | 52.18 [68.29] | 45.78 [45.45] | 28.31 [25.91] | 37.00 [61.88] | 34.99 [36.94] | 100.30 [115.69] |
| Wilcoxon signed rank test | | 0.385 | 0.477 | 0.182 | 0.110 | 0.328 | 0.594 | 0.286 |

A further set of tests was performed to compare the BTX groups with the saline controls (Table 4.20). No differences were seen at the 4 week timepoint. In the 12 week comparisons the medial and lateral thirds of the uninjected side showed a trend for more labeled cells (P= 0.038-0.080 ), as did the proliferative zone (P=0.056). It is clear that mitotic activity of cells in both sides of BTX injected animals is not weaker in comparison with the control at either timepoint. This result indicates that BTX injection not only did not inhibit mitotic activity of cells in the mandibular condylar cartilage but may have stimulated it at least on the uninjected side in the long term.

| Table 4.20. Comparison of BTX with saline controls: Mitotic activity of cells in the mandibular condylar cartilage | | | | | | | |
|---|---|---|---|---|---|---|---|
| Side | Fib Z | Pro Z | M&H Z | Med | Cen | Lat | Total C |
| BTX injected (BTX 4w) | 0.67 [0.68] | 24.93 [21.8] | 24.58 [21.3] | 13.87 [12.2] | 17.67 [18.3] | 19.16 [17.6] | 50.17 [39.4] |
| BTX uninjected (N-BTX 4w) | 0.73 [0.70] | 23.20 [34.1] | 16.27 [16.4] | 12.50 [18.9] | 15.72 [19.7] | 12.02 [12.8] | 40.25 [47.35] |
| Saline uninjected (N-Sal 4w) | 1.09 [0.83] | 19.67 [17.64] | 23.60 [13.35] | 12.77 [7.79] | 19.01 [12.18] | 12.65 [10.88] | 44.35 [26.51] |
| Wilcoxon- Mann-Whitney Rank Sum Test | | | | | | | |
| (BTX 4w) vs (N-Sal 4w) | 0.148 | 0.605 | 0.483 | 0.738 | 0.446 | 0.343 | 0.976 |
| (N-BTX 4w) vs (N-Sal 4w) | 0.343 | 0.563 | 0.088 | 0.148 | 0.166 | 0.563 | 0.232 |
| | | | | | | | |
| BTX injected (BTX 12w) | 1.46 [2.53] | 32.81 [38.23] | 24.30 [23.40] | 21.27 [19.48] | 16.86 [19.63] | 26.82 [34.49] | 58.57 [60.08] |
| BTX uninjected (N-BTX 12w) | 2.30 [3.50] | 52.18 [68.29] | 45.78 [45.45] | 28.31 [25.91] | 37.00 [61.88] | 34.99 [36.94] | 100.30 [115.69] |
| Saline uninjected (N-Sal 12w) | 1.70 [1.90] | 15.70 [17.70] | 26.30 [25.20] | 10.50 [7.50] | 19.90 [25.30] | 13.30 [13.70] | 43.70 [43.80] |
| Wilcoxon- Mann-Whitney Rank Sum Test | | | | | | | |
| (BTX 12w) vs (N-Sal 12w) | 0.238 | 0.230 | 1.000 | 0.656 | 0.882 | 0.456 | 0.710 |
| (N-BTX 12w) vs (N-Sal 12w) | 0.331 | 0.056 | 0.230 | 0.038 | 0.603 | 0.080 | 0.131 |

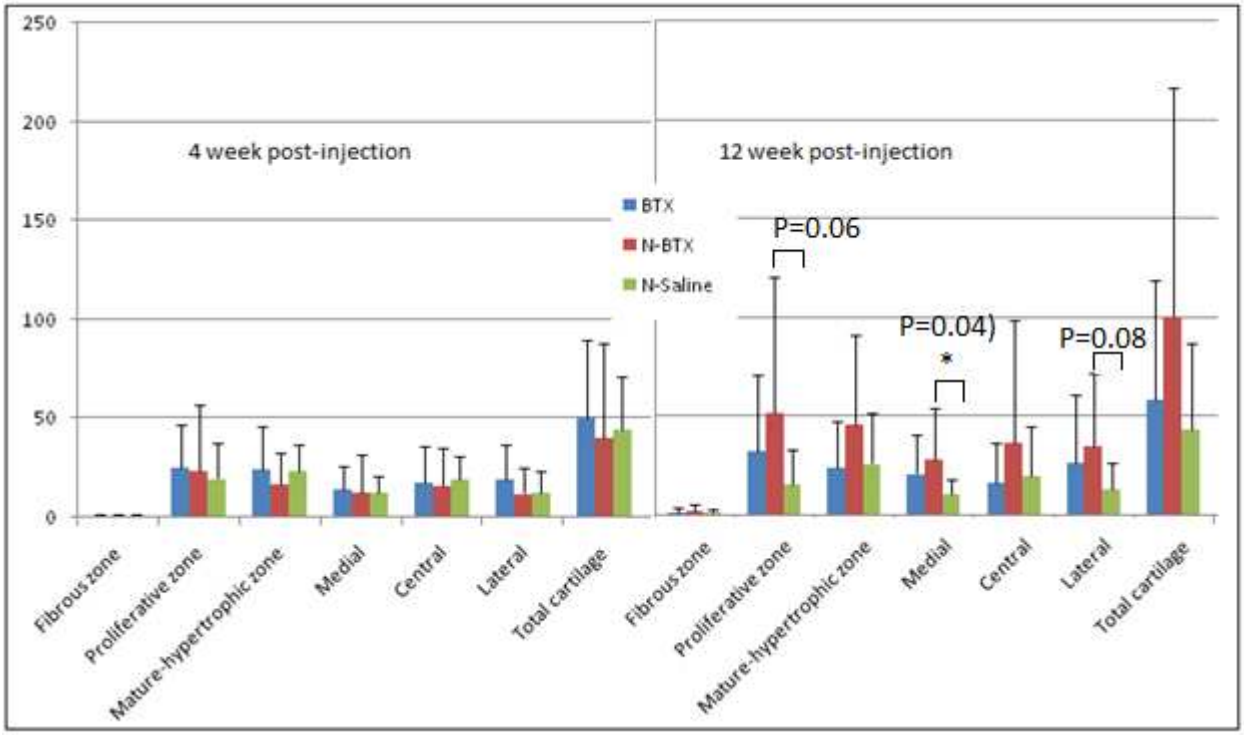


Figure 4.15. Sumary BrdU-positive cells in zones, thirds and in the total condylar cartilage. BTX injected (BTX, blue) and uninjected (N-BTX, red) sides are combined to compare with the saline uninjected control side (N-Saline, green) at 4 weeks and 12 weeks after injection. Labeling in the condylar cartilage in both sides of the BTX treated animals is never less than the control. A trend for higher count at the medial and lateral thirds and in the proliferative zone of BTX uninjected side at 12 weeks timepoint than the control is noted. (Mean, standard deviation)

### 4.8. Difference in mitotic activity between two sides of the BTX injected animals.

The two sides of BTX-treated animals often appeared to be quite different in numbers of labeled cells. However, the side with higher labeling was not consistent. To quantify this observation, the absolute value of the difference between sides was recorded. The results, shown in table 4.25, show that the two condyles of the same animal are significantly more asymmetrical in BTX injected animals than in saline injected animals at 4 weeks and marginally so (P=0.056) at 12 weeks.

| Table 4.21 Absolute value of the difference between two sides of the animals | | |
|---|---|---|
| | 4 weeks | 12 weeks |
| BTX | 45.90 [42.60] | 74.31 [86.23] |
| Saline | 11.36 [10.10] | 24.33 [31.10] |
| Wilcoxon- Mann-Whitney Rank Sum Test | 0.037 | 0.056 |

## 4.9. Mitotic activity in the periostea on the medial and lateral surfaces of the condylar neck

Mitotic activity in the periostea on the medial and lateral surfaces of the condylar necks was similar (Table 4.22). Except for the uninjected side of the BTX 4 weeks group, on average 5-11 cells were labeled per periosteum. The uninjected side of the BTX 4-week animals showed very low periosteal labeling (2-3 cells on average), and table 4.23 shows that this difference was statistically significant. No such discrepancy was seen at 12 weeks.

| Table 4.22 Quantitative analysis of mitotic activity in the periostea on the medial (Med) vs. lateral (Lat) surfaces of the condylar necks in all groups. | | | |
|---|---|---|---|
| Group | Med | Lat | Wilcoxon- Rank Test |
| Sal 4w | 9.01 [9.04] | 9.89[11.8] | 0.735 |
| N-Sal 4w | 9.61[13.05] | 6.76[3.07] | 0.678 |
| BTX 4w | 5.30 [3.3] | 8.20 [9.87] | 1.000 |
| N-BTX 4w | 1.91[1.06] | 3.06[2.98] | 0.575 |
| Sal 12w | 5.59 [6.21] | 6.6 [8.53] | 0.499 |
| N-Sal 12w | 5.38 [5.07] | 5.5 3[3.85] | 0.293 |
| BTX 12w | 10.55 [11.37] | 10.73 [10.84] | 0.594 |
| N-BTX 12w | 6.1 [4.09] | 6.13 [4.99] | 0.953 |

| Table 4.23. BTX treated animals vs the control: mitotic activity of cells in the periostea on the medial and lateral surfaces of the condylar neck (mean[standard deviation]) | | | | | |
|---|---|---|---|---|---|
| Side | | 4 weeks | | 12 weeks | |
| | | Med | Lat | Med | Lat |
| BTX injected (BTX) | | 5.30 [3.33] | 8.20 [9.87] | 10.55 [11.37] | 10.73 [10.84] |
| BTX uninjected (N-BTX) | | 1.94 [1.14] | 3.09 [2.99] | 6.11 [4.09] | 6.12 [4.99] |
| Saline uninjected (N-Sal) | | 9.61 [13.05] | 6.76 [3.07] | 5.38 [5.07] | 5.5 3 [3.85] |
| Wilcoxon-Mann Whitney Rank Sum Test | BTX vs N-Sal | 0.955 | 0.613 | 0.423 | 0.370 |
| | N-BTX vs N-Sal | 0.029 | 0.029 | 0.321 | 1.000 |

# 5. DISCUSSION

Cell replication subserves growth, tissue turnover, and adaptation to new circumstances. Replication of cells in condylar cartilage could be stimulated by growth factors and mechanical loading. In response to increased or decreased strain and loading, a compensatory response may lead to new stable conditions compatible with continued functioning.

## 5.1 Experimental animals and age changes

The animals selected for the experiments have a great influence on the relevance for humans. Although no model will be a perfect mimic of the human, rabbits are considered to be a good model for the human TMJ and these animals have been widely used in many studies on TMJ and masticatory function (Rauch and Hamdy, 2006; Olabisi *et al.*, 2009; de Jong et al., 2011).

Rabbits were all female, so there was no gender effect, and at the start of the experiment the rabbits were all five months old. Although we assumed they were non-growing adults, recent evidence suggests that the rabbit condylar cartilage undergoes histological changes until 13 months of age (Galhardo *et al.*, 2012). Therefore, we could not assume that there would be no age changes between our 4-week post-injection sample (animals about 6.5 months old) and our 12-week post-injection sample (animals about 8.5 months old). Assuming that our rabbits were comparable to those of Galhardo *et al.* (2012), some age changes in the proliferation of cartilage should be expected, notably a decrease.

Our quantitative findings on saline control rabbits showed there was no significant difference in numbers of labeled cells in the overall condylar cartilage from 4 weeks to 12 weeks after injection. Therefore, there was no evidence to conclude that age led to a decrease of mitotic activity of cells in the condylar cartilage in female rabbits between 6.5 and 8.5 months of age.

### 5.2. Cell replication in the BTX treated animals

Kim et al. (2008) investigated the effects of the BTX-A injected into the masseter muscle of a developing rat mandible, using proliferation cell nuclear antigen (PCNA), an antigen that is expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle, as a marker for cell division. Four week old male rats were treated, then sacrificed after 4 weeks. They reported that although the control group had a thicker proliferation zone, higher cell proliferation in comparison with the BTX-A group was not detected. Similarly Matthys (2012), using the same rabbits as in the present study, reported that the thickness of the cellular zone at the apex of the condylar cartilage was reduced, however the present report reveals no diminution of cell mitotic activity in comparison with the other side or with the controls. This result is consonant with Kim *et al*.'s finding that cell proliferation does not account for thinning of the cartilage layer caused by BTX.

In fact the prevailing tendency in the present study was for high (but variable) proliferation rates in the condylar cartilages in animals with BTX-treated masseters. According to Rafferty *et al.* (2012), the BTX-injected side bony condyles were reduced in volume in comparison to saline controls at 4 weeks, while the non-injected side ones tended to be larger in all parameters. If the size of the cartilage was similarly reduced, then the finding of similar quantities of BrdU positive cells in the BTX and saline animals implies that the proliferative index may actually be higher in the BTX condyles.

It is possible that high proliferation in the BTX groups was associated with locations where the fibrous zone showed hypertrophy (Table 4.2), because these locations were often mitotically very active (Figure 4.5). They may represent an adaptive reaction to BTX or a

trauma-repair process. The sporadic occurrence of these locations could also be a factor in the exceptionally high variability of mitotic cell numbers in the BTX animals.

The lack of correspondence between cartilage thickness and cell proliferation seen by us and by Kim *et al*. (2008) suggests that these two parameters are regulated differently by mechanical loading. Cartilage thickness probably depends more on cell size and cell packing than on proliferative activity. Proliferative activity may be promoted rather than inhibited by unloading the condylar cartilage. Ramirez-Yañez *et al.* (2004), investigating the effect of unilateral incisor disocclusion on mandibular condylar cartilage of adult 7 week old rats, reported that after 7 days the numbers of cells immunopositive for BrdU were significantly higher on both sides. Although not verified, this treatment seems likely to have reduced condylar loading. Similarly, Copray et al. (1985) reported that organ culture of rats showed that under the influence of a continuous compressive force smaller than 0.5 g, the incorporation of [3H]-thymidine in the cartilage was significantly raised, whereas continuous compressive forces above 0.5 g and intermittent compressive forces (0.7 Hz) reduced the proliferative activity in the cartilage compared to the uncompressed *in vitro* controls. Thus there are two possible explanations for continued, and possibly elevated, high proliferations in the condylar cartilage of BTX-treated rabbits: decreased loading or response to trauma. Of these, a response to trauma seems more likely. Strain results on the same BTX-treated animals suggested that the condyle on the BTX-uninjected side was not under decreased loading at any time, and that although the condyle on the BTX-injected side was underloaded 4 weeks postinjection, loading had recovered in the 12-week group (Rafferty et al., 2012). Thus loading does not explain high proliferation rates on both sides and at both timepoints. In contrast, local thickening of the cartilage to fill bony defects was seen on the two sides and at the two timepoints (Table 2) and furthermore

accounts for high variability, because many of the condyles listed in Table 4.2 were the same as these showing abnormally high numbers of labeled cells in Tables 4.4-4.11. A similar abnormality was seen in two animals of the untreated control groups (#6683 and #6684), and in both cases, the labeled cell count was high (77 and 84, respectively). The fact that the abnormality occasionally existed in normal rabbits implies that this sort of damage can occur spontaneously. Finally, unlike the saline controls, the condylar cartilages of the BTX-treated rabbits tended to show more mitoses 12 weeks post-injection than 4 weeks post-injection, especially so on the uninjected side. This must be a treatment effect rather than an age effect, since it was absent from controls. Most likely, this presents continued recovery from the abnormal functional situation caused on both sides by BTX injection into one masseter.

### 5.3. Distribution of BrdU labeled cells in the zones of the mandibular condylar cartilage

The low labeling of the fibrous zone showed that any thickening of this zone was not due to increased recruitment of newly divided cells.

Visnapuu *et al.* (2000) using normal rats 28 days (young) and 70 days (mature) old, reported that at 28 days, mitoses were seen under the fibrous zone, and the mitotic area was negative for type II collagen. However, at 70 days, a narrow layer of proliferative cells had collagen type II matrix. Because collagen type II is considered to be a marker for identification of typically mature cartilage cells, these findings showed that in young animals, condylar cartilage behaved more like a growth cartilage. However, in mature animals, its function shifted to an articular cartilage.

The interpretation of zone pattern thus depends on whether the condylar cartilage is considered to be a growth cartilage or an articular cartilage. Because at the baseline, the 5 month

old rabbits were basically full- grown, it is probably best to consider the primary function of the cartilage as articular. At the 4 week and 12 week timepoints, the rabbits were about 6.5 and 8.5 months old and would be expected to show similarity in labeling. This was generally true for the comparison of the saline-treated animals at the two timepoints. The fibrous zone always had very low labeling, whereas labeled cells were common not only in the proliferative zone but also in the mature-hypertrophic zone, where they were sometimes more numerous (Table 4.14). This interesting finding implies either that many cells had been translocated inferiorly in the 7 days between BrdU administration and sacrifice (which implies that growth was still occurring) or that the maturing chondrocytes were still actively dividing. Like the saline controls, the BTX-treated animals showed few labeled cells in the fibrous zone but many in both the underlying zone. Here there appeared to be a slight preponderance in the proliferative zone that may have resulted from the higher frequency of abnormal fibrous layer thickening (e.g., Figure 4.4).

**5.4. Distribution of BrdU labeled cells in the thirds of the mandibular condylar cartilage**

Rafferty *et al*., (2012) reported that in the sagittal plane, at both sides of the saline treated animals, the condylar neck showed similar orientations of strain with compression directed vertically regardless of chewing side. However, for injected side of BTX treated animals at 4 weeks, resulting strain levels on the condylar neck tended to be low, and compressive strain on the condylar neck was abnormally oriented from “anteroinferior to posterosuperior”. Unfortunately, these findings shed little light on load distribution in the coronal plane, which was the plane of study in the present work.

Our results suggested that proportional labeling may have increased in the lateral third of the condylar cartilage on the injected side of the BTX injected animals (Figure 4.14). This is in

contrast to the uninjected side, which continued to show a plurality of labeled cells in the central third even at 12 weeks, when the absolute number of labeled cells was elevated at the medial and lateral thirds (Table 4.20). Because the distribution of stress within the articular surface is unknown, it is unclear why this occurred. However, if proliferation is related to loading, this finding, if real, points to a change in direction of load as an effect of BTX paralysis. Specifically, it suggests that load on the BTX-injected side became directed laterally rather than centrally. Also, medial pterygoid muscle changed, compensated at least in part for the BTX-paralyzed masseter.

**5.5. Differences in mitoses between the two condyles of the BTX treated animals.**

Our data showed that mitotic activity in condylar cartilage at two sides of the same BTX injected animal at either timepoint was often quite different. The two condyles of the same animal were more asymmetrical in BTX injected animals at 4 weeks than in saline injected animals (P=0.037). However, this asymmetry was not quite statistically significant at 12 weeks (P=0.056).

The previous study on these animals (Rafferty *et al*., 2012; Matthys, 2012) indicated that the condyles of the BTX group were asymmetrical in many features, but always in the same direction. Injected side bony condyles were smaller and less dense than uninjected side condyles. The uninjected side condyles were similar to both sides of saline control except for a tendency to be larger overall. This tendency to overgrowth could be the reason that some uninjected sides of BTX animals showed high condylar cartilage labeling. However, the sporadic nature of asymmetrical labeling is more likely due to the sporadic damage that was frequently seen on both sides of the BTX groups.

### 5.6. Distribution of BrdU labeled cells in the periostea on the medial and lateral surfaces of the condylar neck

Compared to the BTX-treated side and the saline controls, the uninjected side of the BTX 4-week animals had very low periosteal labeling. Paradoxically, this finding might be correlated with enlargement of the uninjected condyles at this timepoint (Rafferty et al., 2012). The condyle grows according to “Enlow’s V- Principle”, in which the medial and lateral poles actually resorb as the condylar cartilage grows. At 12 weeks neither enlargement (Rafferty *et al.*, 2012) nor unusually low labeling (Table 4.28) persisted.

## 5. CONCLUSION

Our findings showed that BTX-A therapy affected mitotic activity of cells at the mandibular condyle. BTX caused a high frequency of local thickening of the fibrous zone associated with high proliferation. Overall levels of proliferation were comparable to or higher than those of controls. The effect was especially prominent on the uninjected contralateral side 12 weeks after BTX administration.

### Future direction

The future direction to assess effects of BTX treatment on mandibular condylar cartilage would be to design and perform studies about the distribution of stress within the articular surfaces. Investigation at different ages and at other time points (2 weeks, 3 weeks, 16 weeks, 20 weeks…) should be performed. It will be necessary to detect apoptosis and the activity of osteoclasts and chondroclasts. It would be helpful for clinical practice to design studies comparing BTX therapy at single- dose vs multi-dose.

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