Suburothelial myofibroblasts in the human overactive bladder and the effect of BoNTA treatment

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19 **Abstract**

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22 overactivity, Botulinum toxin A

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1. Introduction

Recently, a novel cell type, the so-called suburothelial myofibroblast (MF), has been identified in the lamina propria of the bladder wall. These cells form a functional syncytium through extensive connexin43 (Cx43) gap junction coupling. By their close proximity to afferent nerves and the fact that their own activity is modulated by exogenous agents, it is proposed that these cells act as modulators of afferent bladder sensation, and are able to integrate focal signals from different regions of the bladder wall[1], [2]. In this context, novel aspects have been added to existing concepts accounting for the pathogenesis of bladder overactivity (OAB): It has been proposed that changes in the sensitivity and coupling of the urothelial-myofibroblast network leads to an enhancement of spontaneous contractions . Modulating the coupling instensity between the cells would have an impact on signal propagation within the syncytium, and consequently the number of Ad –fibres stimulated [3].
The concept of gap junction remodeling is well established in cardiac research: Remodeling of Cx43 gap junction distribution and expression has been described in ischemia, infarction, and dilated cardiomyopathy [4-6] and is a potentially significant contributor to the arrhythmogenicity of cardiac disease [7-9]. Recent animal studies have provided increasing evidence that gap junctions play a role in the generation of unstable bladder condition: In a rat model of detrusor overactivity (DO) induced by spinal cord transsection [3], a marked up-regulation of suburothelial gap junctions was demonstrated. Moreover, gap junction blockers were capable of reducing spontaneous bladder contractions. In contrast, in a preliminary study [10] of 7 patients with urge symptoms, but no urodynamically confirmed DO, a trend towards increased suburothelial gap junction formation compared to normal controls failed to

reach statistical significance. A study with sufficient numbers of clinically well defined patients is lacking to date.

Over the last 10 years, patients with intractable DO of neurogenic (NDO) or idiopathic (IDO) origin have been successfully treated with intradetrusor injections of BoNT/A. Placebo-controlled trials [11-14] have confirmed its impressive efficacy with symptomatic and urodynamic improvements [15, 16].

It has been hypothesised that BoNT/A injected in the overactive human bladder has a complex inhibitory effect on urothelium/suburothelium dependant afferent pathways, which are important in mediation of intrinsic or spinal reflexes thought to cause DO [17]. Extending the hypothesis that the suburothelial myofibroblasts act as integrating stretch-receptor organ, an effect of BoNT/A on gap junctions in these cells might result in their reduced activation and electrical coupling. Mediation of afferent signalling between the urothelium and the closely apposed nerve endings would thus be reduced, achieving maximisation of the BoNT/A-induced peripheral desensitisation.

We aimed to examine for the first time a possible role of suburothelial MFs in human neurogenic or idiopathic DO and whether the action of BoNT/A in human DO is partly exerted through an effect on suburothelial MFs. To do this, we studied the immunohistochemical expression of Cx43, vimentin and c-kit (markers of MFs) in patients with NDO/IDO before and after treatment with BoNT/A and in comparison with controls.

2. Methods

2.1 Patients

A total of 21 consecutive patients (17 women, 4 men, mean age ** years) from a group of patients with urodynamically proven refractory DO were treated according to a research protocol approved by the local Research Ethics Committee. Intradetrusor injections of BoNTA (Botox®, Allergan Ltd) were delivered by a minimally invasive outpatient technique using a flexible cystoscope [18]. Patients with NDO received 300 units of Botox®, while those with IDO received 200 units, injected at 30 and 20 sites respectively, avoiding the trigone [11]. 10 patients (8 women, 2 men, mean age 48.2+-5.9 years, range 42 to 60) had NDO, 11 patients (10 women, 1 man, mean age 48.2+-15.9 years, range 19 to 68) had IDO. Flexible cystoscopic bladder biopsies were obtained at baseline pre-treatment, and during check flexible cystoscopy, 4 and 16 wk after each treatment session. Biopsies were obtained from a consistent bladder area, 2 cm above and lateral to the ureteric orifices [19]. Control tissue was obtained endoscopically from 10 patients (8 women, 2 men, mean age 52.7±13.4 years, range 31 to 72) being examined under anesthesia prior to pelvic floor repair procedures, who had macroscopically normal bladders, no symptoms of bladder overactivity, and sterile urine at the time of endoscopy.

2.2 Immunohistochemistry

All histology biopsy specimens were snap-frozen in liquid nitrogen, embedded in optimal cutting temperature compound, and kept at -60°C, until frozen. 2 x 3 sections (10µm) per specimen were cut in a cryostat and collected on superfrost

aminopropyltriethoxysilane-coated slides. Sections were post-fixed in methanol at -20°C for 5min, rinsed twice in PBS and blocked in 1% BSA for 45min before incubation with the primary antibodies for 2h at RT. For quantitative immunofluorecence, 3 sections per specimen were co-labelled for vimentin (rabbit polyclonal, Abcam, ab 7783-500; 1:100) and Cx43 (mouse monoclonal, Chemicon, MAB 3067; 1:1000), 3 further sections for c-kit (mouse monoclonal, NovoCastra, NCL-cKIT; 1:100) and Cx43 (rabbit polyclonal, Invitrogen, 71-0700; 1:500). For Cx45 labelling (kind donation of Prof NJ Severs, Imperial College, London), sections were incubated over night at RT. Binding sites were visualised using Cy3- and FITC-conjugated secondary antibodies (Cy3, goat anti mouse, Chemicon, AP181C; 1:500; FITC, donkey anti rabbit, Chemicon, AP182F; 1:50); nuclei were counterstained with DAPI (Invitrogen, D1306, 1:50.000) during incubation with one of the secondary antibodies. Slides were coverslipped using Citifluor Mounting medium (Agar Scientific), and immediately photographed. Immunolabelled sections were examined using a laser scanning microscope (Zeiss LSM-510 Meta, Germany) equipped with an argon laser (458nm, 488nm, 514nm), a helium-neon laser (543nm, 633nm) and a 405 nm diode laser, using a x40 oil-immersion objective. Fluorescence was excited at 488nm (Cy3), 405nm (DAPI) and 543nm (FITC) and recorded with separate detectors. Multitrack scanning avoided 'bleeding through' of the fluorescence in doublelabelling experiments. 3 images per section (areas with highest immunoreactivity) were taken in a blinded fashion, rendering 9 representative images of the suburothelial gap junction-network per biopsy for analysis. To ensure comparability of fluorescence signal intensity between the samples as well as comparability between this and previous studies [3, 10], we first calibrated the

detection system on a reference section and re-used the parameter settings (pinhole, optical slice>1µm; detector gain) for all images.

2.3 Quantitave analysis and statistics.

For examining the amount of Cx43 labelling, a square section of 75x75µm, representing the minimal extension of the Cx43 positive band in a transversally cut section, was cropped from the Cx43-positive area of each image and analysed using ImageJ software (http://rsb.info.nih.gov). Determination of gap junction density was preferred to an estimate of the extension of the Cx43 positive band, as it was impossible to regulate the cutting plane for each biopsy in order to obtain a strictly transversal section. Only the monoclonal mouse Cx43 antibody was used for quantification, as it showed a much more intense and reliable staining than the polyclonal rabbit antibody. Colours were split to render images for the Cx, nuclear, or smooth muscle labels alone (Fig2). The images were then converted into black-and-white bitmaps after constant setting of threshold levels, with gap junctions now being represented by single black particles which were automatically counted at set parameters. The number of particles was additionally expressed as a ratio of the number of nuclei to normalise for any variation in cellular component in different sections. For the numerical determination of c-kit positive cells within the Cx43 positive band, a slightly larger section (120x120µm) was cropped to obtain higher numbers and more accurate results, given the sparse distribution of this cell type. Vimentin-labelling was not considered suitable for quantification, as it represented a rather diffuse, ill-defined staining, compared to Cx43 and c-kit. Quantitative data are shown as mean±SEM. Differences between data sets were tested with Student's *t*-

142 test (unpaired for baseline vs. control, paired for baseline vs. 4 weeks vs. 16 weeks);
143 the null hypothesis was rejected when $p<0.05$.
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3. Results

3.1 Localisation of immunolabelling

A band of strongly vimentin positive cells was seen immediately below the urothelium, lying in rows, with their cell bodies and long projections parallel to the basal lamina (Fig. 1a). Cx43 immunolabelling was extensively distributed in the suburothelial layer, coinciding with the layer of vimentin positive cells, and sometimes slightly offset towards the detrusor layer, possibly suggesting that gap junctions, located on the cell filaments, projected somewhat away from the urothelium. The boundary of the Cx43/vimentin positive band was more sharply defined on the urothelium-facing side than on the submucosal side where the labelling density tended to decrease progressively with depth. Cx45 immunolabelling was not detectable in the suburothelium.

C-kit labelling showed a sharp cellular staining of outstretched, spindle-shaped or stellate cells, loosely scattered across the whole suburothelium, a proportion being located within the vimentin/Cx43 positive band (Fig. 1b). Direct contacts between c-kit positive cells were only rarely observed, but regularly with a punctuate Cx43 positive staining interposed (Fig. 1c). Interestingly, there was only poor co-localisation of c-kit and vimentin, the latter being present in the distal processes of ckit positive cells (Fig. 1d).

3.2 NDO vs. IDO vs. controls and effect of BoNTA

There was a significant, two-fold upregulation of gap junction density in both IDO and NDO patients compared to controls (Fig. 3a). The same results were obtained, when the number of gap junctions was correlated to the nuclear count (controls vs. NDO:

0.8±0.09 vs. 1.7±0.2), discounting the possibility that results might be confounded by various levels of section integrity. However, there was no return to control values 4 and 16 weeks after BoNTA injection in both groups. The sharp cellular c-kit labelling was equally suitable for quantitative analysis. Mast cells potentially express the c-kit receptor, but they were easily recognised by their large, round cell bodies and large, round nuclei and excluded from analysis. Further, co-labelling with Cx43 enabled us to reliably determine the number of c-kit positive cells within the Cx43 positive band. In contrast to gap junction density, there were no significant differences between controls and NDO or IDO groups, respectively, and no changes were detected after BoNTA treatment.

No differences could be identified between NDO and IDO baselines for both c-kit and Cx43.

4. Discussion

This is the first study, to the best of our knowledge, to examine the role of suburothelial MFs in the neurogenic or idiopathic overactive human bladder. Using immunofluorescence we have found increased presence of the gap junction protein Cx43 in the suburothelium of both patient populations in comparison with controls, suggesting that increased gap junction formation in the suburothelium could have a causative association with human DO.

4.1 IR characterisation of MFs

Suburothelial MFs have first been characterised by electron microscopy, mainly on the basis of a fibronexus, intracellular stress, elongated processes, abundant vimentin intermediate filaments, extensive smooth ER, dense bodies, and the presence of an interrupted basal lamina [1] [20]. The identification of human MFs by immunohistochemistry remains controversial. As yet, no diagnostic marker has been identified that is predictably expressed by all MFs, and by no other cell type. A previous combined EM and immunofluorescence study [21] found MFs to stain intensively for vimentin, but only poorly for c-kit and not at all for alpha-SMA. *Because vimentin filaments are found in MFs and fibroblasts but not in SMCs, vimentin antibodies are a useful tool with which to distinguish between MF candidates and SMCs, although fibroblasts cannot be excluded.* The cells also expressed abundant label for the gap-junctional protein, Cx43, but were immunonegative for Cx40 and Cx45. The Cx43 immunofluorescence represented gap junctions between myofibroblasts, preferentially located on deeply penetrating branching processes, as was confirmed by the specificity of electronmicroscopic immunogold labelling. Alpha-SMA as a useful marker for cell definition has been ruled out also by others [20, 22], whereas c-kit has repeatedly

been used to identify myofibroblasts. C-kit is a proto-oncogene encoding the receptor tyrosine kinase. The ligand for kit (NCL-cKit) is stem cell factor. Kit signalling is important for the development and survival of structurally related interstitial cells in the gut. However, c-kit-expression might be down-regulated in the stable adult cell line. In the guinea-pig suburothelium, c-kit labelling reveals a network of interconnected stellate cells with many branches. There is an evident co-localisation with vimentin, although many vimentin-positive cells - equally branched and interconnected – were kit negative [23]. In the suburothelium of the human bladder however, c-kit positive cells show a much more scattered and sporadic distribution [24, 25], making a possible network formation rather unlikely. No staining for vimentin was performed in these studies.

Our labelling is in line with previous reports. Vimentin-positive cell bodies with typical processes form a layer immediately underneath the urothelium coinciding with a dense band of Cx43, sometimes slightly offset towards the detrusor layer, reflecting the fact that the gap junctions, which are located on the cell filaments, project somewhat away from the urothelium. C-kit positive cells show the characteristic outstretched, spindle-shaped cell bodies and are located within the band of vimentin- and Cx43-positive cells. However, this cell population seems too sparsely distributed to form a network, and direct contacts between single cells can be observed only occasionally (but usually with an interposed gap junction). Also, there is only poor co-localisation of c-kit and vimentin which at best is present in the very distal branches of c-kit positive cells. The discrepancy between c-kit and vimentin-labelling has always been a matter of controversy [21, 23]. Some argue that only a subpopulation of SM express c-kit. At the end of cell growth when such cells have

fully differentiated from mesenchymal cells they lose their reactivity to c-kit. However, that does not explain the weak staining for vimentin of c-kit positive cells.

4.2 Increased gap junction coupling in OAB

Several recent studies in animals and humans have suggested an involvement of MFs and/or interstitial cells in the generation of the OAB syndrome. In the human detrusor found c-kit positive cells on the boundaries of muscle bundles were significantly increased in specimen from patients with either IDO or NDO compared to controls[26]. In guinea-pigs with bladder overactivity induced by BOO, Kubota et al. [25] demonstrated an increased density of c-kit positive cells, and an altered, more wide-spread distribution of vimentin-positive cells in the suburothelial space when compared with controls. Several studies [27-29] have shown an overall increase of connexin43 transcript and protein in the overactive and/or obstructed rat bladder, however, without structural localisation. A rat model of OAB showed significantly higher suburothelial cx43 immunoreactivity, and gap junction blockade reduced spontaneity, and it was proposed that spontaneous activity in the bladder requires gap junction upregulation in lamina propria myofibroblasts [3]. A study of the human overactive detrusor [30] found up-regulated Cx43 mRNA and a denser Cx43 staining pattern, however, localising these gap junctions to the cytoplasmatic membranes of smooth muscle cells. Similarly, Neuhaus et al. [10] showed significantly higher Cx43 expression in the detrusor muscle and a tendency to higher Cx43 expression in the suburothelial layer to be associated with idiopathic urge symptoms. However, the study population was heterogeneous, comprising patients with urge incontinence, mixed incontinence, and painful bladder syndrome with no reported urodynamical confirmation of DO.

The present study was performed with tissue from 8 controls and 21 patients with cystometrically confirmed, refractory, IDO and NDO. In consistence with the literature, we found the suburothelium to be immunopositive for Cx43, but immunonegative for Cx45. We found a significant increase of about twofold control in both IDO and NDO. Interestingly, this was not accompanied by increased numbers of c-kit positive cells, nor was there a return to normal values after BoTNA treatment. The strategic position of MFs directly beneath the urothelium suggests they are a link between urothelial signalling during bladder filling and afferent fibre stimulation. They elicit spontaneous electrical and intracellular Ca^{2+}-responses and also respond to exogenous agents, such as ATP, and low pH-agents postulated to be transmitters of sensory responses in the bladder [31, 32]. Moreover, cell functions are augmented through physical interaction with their neighbours. Signals initiated in one group of cells can be transmitted through considerable distances in the suburothelial layer [2]. Modification of the coupling characteristics of the suburothelial syncytium may have profound impact on bladder sensation and, thus, play a role in the development of urgency and detrusor overactivity. Although BoNTA injected into the bladder wall has been known to reduce the pathological sensation of urgency and suppress DO, the density of suburothelial gap junctions does not seem to be altered by intradetrusoral BoNTA injections. Thus, the suppression of DO by BoNTA might not be exerted via a remodeling of the Cx43 gap junction distribution, at least in the suburothelium. It seems plausible that Cx43, as a structural surrogate, is not affected in its density by BoNTA treatment, although altered release of transmitters might indirectly influence gap junction function, even though the gap junction proteins may not be changed. It has been shown to decrease sensory $P2X_3$ and TRPV1 receptors in suburothelial

nerve fibres [33], which might represent the structural surrogate mainly affected by BoNTA in the suburothelial space.

4.3 Methodological considerations

The authors are well aware of the general methodological restrictions of quantitative immunohistochemistry. It is widely agreed that statistical differences of 25 % or lower are to be interpreted with caution due to the restricted accuracy of the method. Furthermore, areal or fibre-like staining is problematical as different cutting planes or angles might result in different countings in the same specimen. For example, a single nerve might be depicted as a singular dot or as multiple fibre-like structures depending on the course it takes in relation to the cutting plane. Finally, staining and analysis procedures have to be kept as standardised and automated as possible. We are quite confident that the present study reflects these considerations to the maximal possible extent. First, we restricted our analysis to clear punctuate staining, that is gap junctions with a diameter of 0.1 µm [10], or to c-kit positive cells that due to their scattered distribution were easy to count. Second, we applied the same protocols for tissue processing, staining and analysis throughout. It has been reported that MFs become increasingly numerous toward the bladder neck [24], so we took care that biopsies were constantly taken from the same area. The fact that the punch biopsies were obtained the same way as the BoTNA injection was applied, namely via flexible cystoscopy without anaesthesia, naturally restricted size (regularly less than 1 mm^3) and quality of the biopsy; it would have been unethical to subject the patient to general anaesthesia and rigid cystoscopy just for the sake of research. Several studies already published [33, 34] have been carried out using these specimen, and it will, for the same reasons, be the only kind of tissue available for future research in this field. However, we were convinced that the level of tissue preservation was sufficient to serve our purposes. Imaging and analysis were blinded and fully automated with constant parameter settings throughout as has been done in previous studies. To ensure that the analysis was not confounded by any kind of squeezing or twisting of the punch biopsy, we additionally correlated the number of gap junctions to the nuclear count and obtained identical results.

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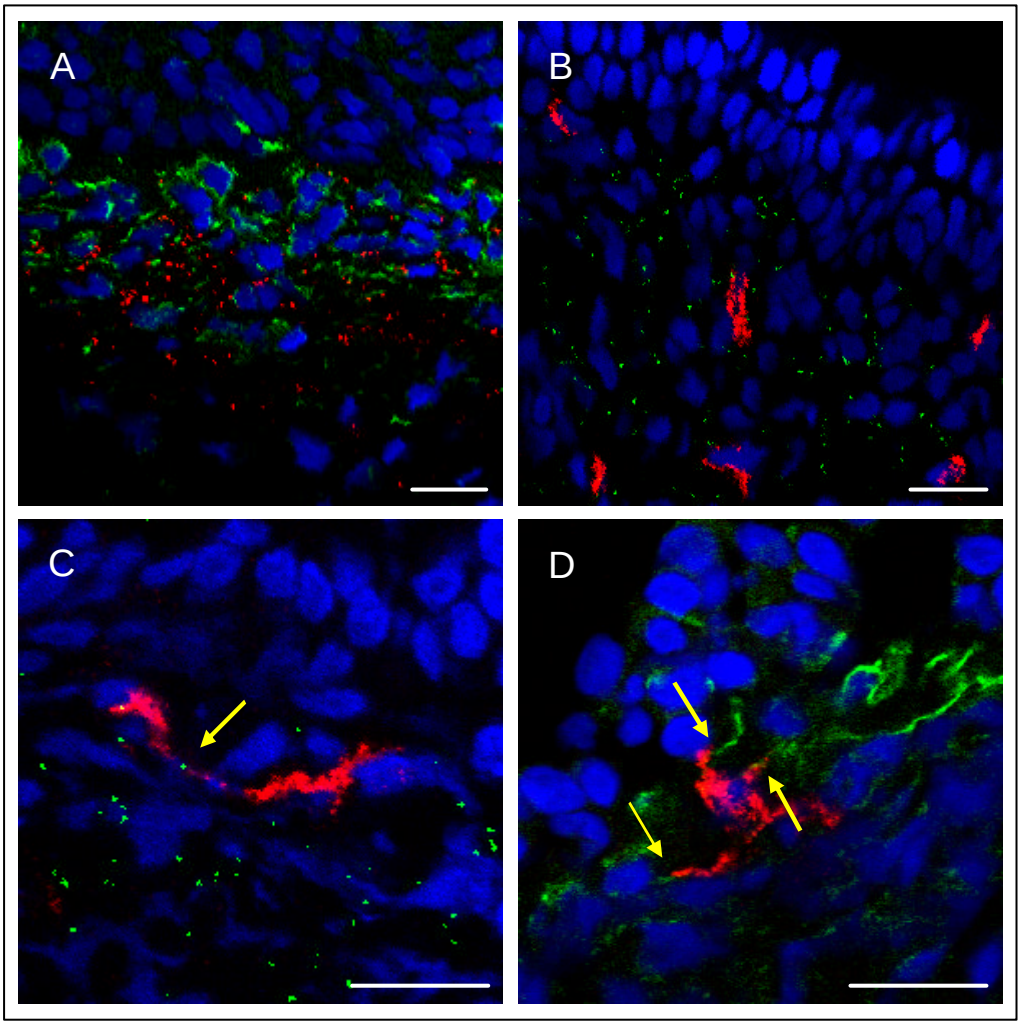
Figure legends

Fig 1. Cross section of bladder urothelium and lamina propria; **A:** green: vimentin, red: Cx43; **B and C:** red: c-kit; green: Cx43; **D:** red: c-kit, green: vimentin. Bar 20 µm.

Fig 2. Illustration of the quantification process of suburothelial Cx43 immunofluorescence.

Fig 3. Summary of the quantitative analysis of Cx43 (**A:** IDO, **B:** NDO) and c-kit (**C:** IDO, **D:** NDO) immunofluorescence in biopsies from controls and IDO and NDO patients pre and post BoNTA treatment (4 and 16 weeks); data given as mean±sem; * $p<0.05$, ** $p<0.005$.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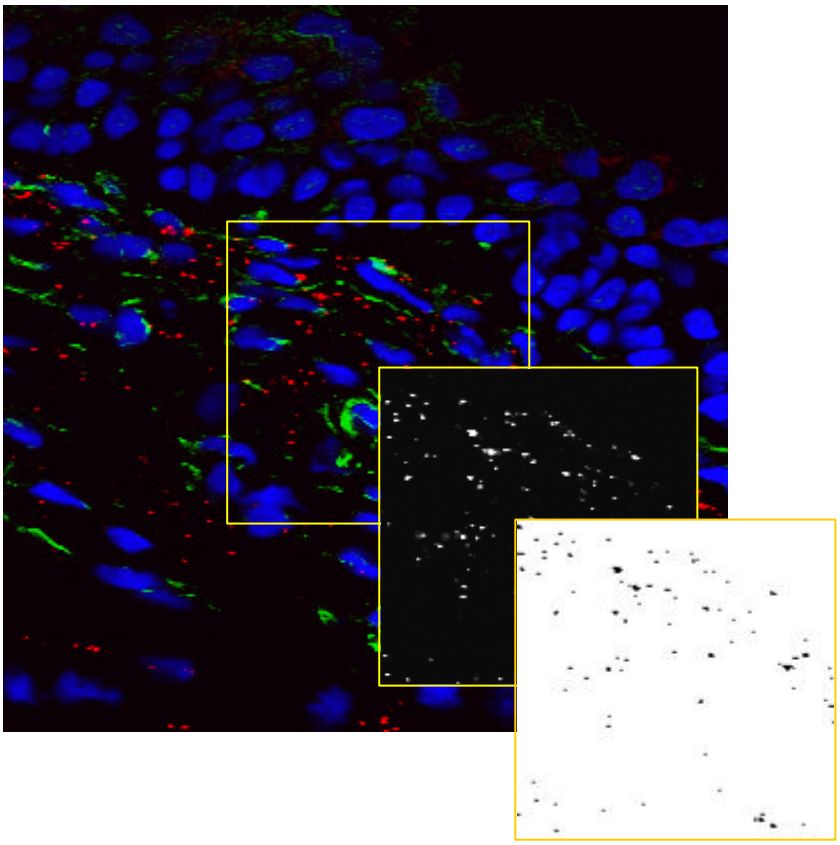

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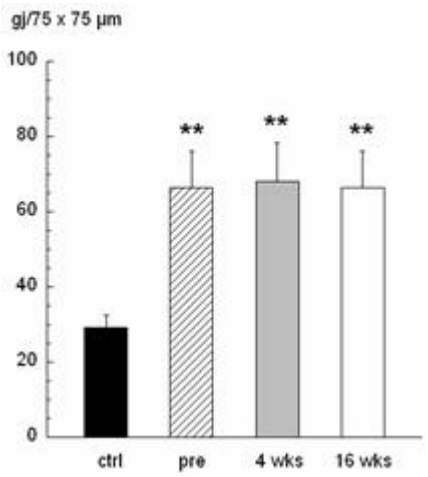
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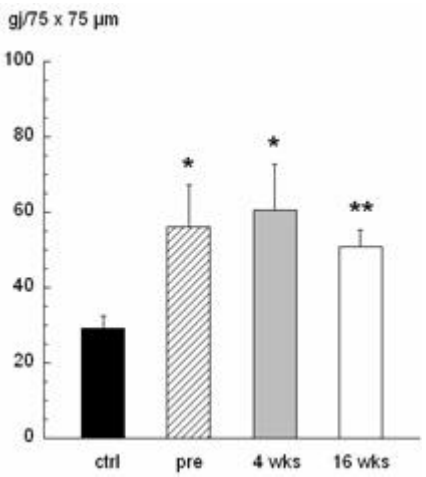
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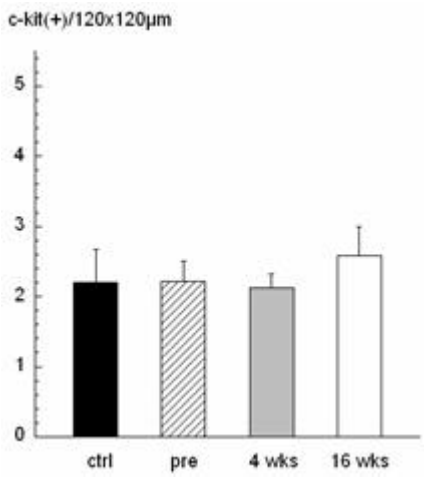
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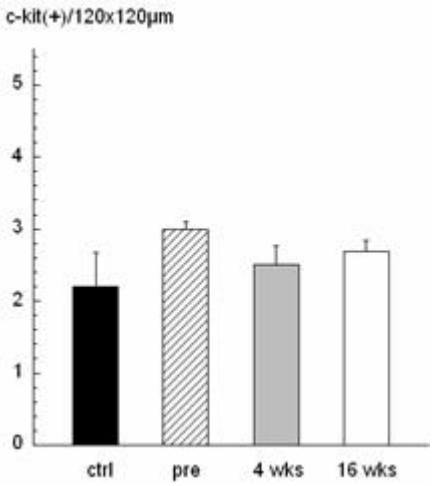
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