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Application of *Clostridium* spp. and their toxins/enzymes in treatment of oncologic and other pathologies

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ABSTRACT

Clostridium is a genus of Gram-positive bacteria including several significant human pathogens. Bacteria are obligate anaerobes capable of producing spores. Bacterial toxins are responsible, among others for botulism, tetanus, gas gangrene and gastrointestinal tract infections. However, after years it turned out that bacteria of the *Clostridium* genus, their toxins and enzymes may be used in modern medicine. Botulinum toxin nowadays is used commercially for several medical and cosmetic purposes. Collagenase *Clostridium histolyticum* (CCH) is an enzyme, that dismantles collagen. CCH is used as for the treatment of connective tissue diseases such as Dupuytren's contracture and Peyronie's disease. Bacteria from *Clostridium* genus and their toxins and enzymes found also application in oncology. In 1813, Vautier reported regression of cancer in patients with gangrene, caused by *Clostridium perfringens*. Hypoxia is a pathophysiological feature in the majority of solid tumours. Research show also that it is possible to use *Clostridium* spp. for biotechnology purposes in oncology. A recombinant produced C-terminus of the *C. perfringens* enterotoxin was conjugated to gold Nanoparticles to produce a C-CPE-AuNP complex, which can be later used for killing tumor cells with laser. Similarily the *C. butyricum* strain has been successfully used in the therapy of colorectal cancer in the mouse model. It can reduce the expression of inflammatory cytokines, which are vital in carcinogenesis process. Also *C. novyi* – NT with iron oxide found application in modern imaging of solid tumors in Magnetic Resonance Imaging (MRI). Also *C. novyi*- NT spores can be successfully labeled with iron oxide nanoparticles for MR imaging. Bacteria of the genus *Clostridium* have been an important clinical problem for centuries. In this review we will discuss the application of *Clostridium spp*. and their toxins/enzymes in medicine, especially in treatment of oncologic and other pathologies in the light of medical literature, as well as our own experiences.

INTRODUCTION

Clostridium is a species of Gram-positive obligate anaerobes, capable of producing spores. The bacterial reservoir is soil and digestive system of human and animals.The bacterial malignancy factors are toxins, produced most often in the intestinal lumen, wounds or soft tissues. Bacterial toxins are responsible, among others for botulism (*C. botulinum*), tetanus (*C. tetani*), gas gangrene and gastrointestinal tract infections (*C. perfringens*) and symptomatic *Clostridioides difficile* infections (CDI), which can cause symptoms such as abdominal pain, diarrhea and fever. This is also the most common cause of antibiotic associated diarrhea. Clostridial infection may be lethal. In this review we will discuss the application of *Clostridium spp*. and their toxins/ enzymes in medicine, especially in treatment of oncologic and other pathologies in the light of medical literature.

HISTORY: WHAT WE KNOW ABOUT DEVELOPMENT CLOSTRIDIAL INFECTIONS?

Through ages clostridial infections causing gas gangrene were serious treat for surgeons. But it wasn't until the first half of the 20th century that William Welch discovered the etiological factor of gas gangrene. Welch was an American physician, pathologist and bacteriologist. He was precursor of modern medicine named one of the "Big Four" founding professors at the Johns Hopkins Hospital and also the founder of the Johns Hopkins School of Hygiene and Public Health, the first School of Public Health in the USA. Welch's research was principally in bacteriology, and he is the discoverer of the microorganism that causes gas gangrene. It was named *Clostridium welchii* in recognition of that fact, but now the microorganism is known as *Clostridium perfringens*.

C. botulinum was first recognized and isolated in 1895 by Emile van Ermengem from home – made ham associated with a botulism outbreak. The isolate was originally named *Bacillus botulinus*, after the Latin word for sausage, *botulus* ("sausage poisoning" was a common problem in XVIII/XIX century Europe most likely caused by botulism toxin).

First descriptions of tetanus in Hippocrates' Aphorisms dated to IV century BCE. In 1889, *C. tetani* was isolated from a patient by Kitasato Shibasaburō. Moreover he later proved that the disease is caused by toxins produced by bacteria.

C. histolyiticum was first isolated in 1916 by Weinberg and Séguin. They discovered bacterial cultures may cause extensive local

tissue destruction, splitting of the skin and sometimes autoamputation in human body. In 1923 Bergey, Harrison, et al. reclassified it as *Clostridium histolyticum.*

Clostridium butyricum is uncommonly reported as a human pathogen and is widely used as a probiotic in Asia (particularly in Japan and China). First strains were isolated in the 1930s in Japan from soil. For over 60 years *C. butyricum* was used as a probiotic in Japanese hospitals, especially during an antibiotic therapy with strong antibiotics (eg III generation cephalosporins or Levofloxacin).

Clostridioides (Clostridium difficile was first isolated from the stool of a healthy infant by Hall and O'Toole in 1935. It was not recognized as a pathogen until 1978, however, that George and colleagues associated *C. difficile* with human disease and discovered that *C. difficile* was the microorganism responsible for the majority of cases of anti-biotic-associated diarrhea in the developed countries. The species was transferred from the genus *Clostridium* to *Clostridioides* in 2016, thus giving it the binomial *Clostri-dioides difficile*. This new name reflects the differences in taxonomy between this species and members of the genus *Clostridium*.

MEDICAL APPLICATION OF *CLOSTRIDIUM* SPP. AND THEIR TOXINS/ENZYMES

However, after years it turned out that bacteria of the genus *Clostridium*, their toxins and enzymes are used not only in cosmetic purposes but in modern medicine as well. We currently use clostridial toxins and enzymes, but also biotechnologically modified microorganisms for clinical purposes.

Botulinum toxin (BTX), produced by the bacterium *Clostridium botulinum* and related species, is a neurotoxic polypeptide protein. It prevents the release of the neuro-transmitter acetylcholine from axon endings at the neuromuscular junction and thus causes flaccid paralysis in mechanism of localized reduction of muscle activity by inhibiting acetylcholine release at the neuromuscular junction. In 2004, the US Food and Drug Administration approved its application in the treatment of various medical conditions, such as facial wrinkles, strabismus, cervical dystonia, blepharospasm, and hyperhidrosis. Since then crowds of patients could take advantage of this method. The toxin nowadays is more widely used both for medical and cosmetic purposes. In neurology it is used for treatment of disease's connected with muscle spasticity such as head and neck (dystonia), vocal cords and jaw spasm. Dystonia is a condition characterized by involuntary muscle contraction in one or more regions. It may be idiopatic or secondary to other neurologic conditions. Many patients complains the pain accompanying the disease. Current medical strategies consist of oral treatment, behavioral modification of lifestyle, exercises and BTX injection, where intermittent injections were found superior to the above treatment (Grazzi, 2014). It is not only effective and well- tolerated treatment but also BTX reduces concomitant pain.

Also chronic migraine (CM) reductant for other treatment methods is an indication for botulinum use. CM affects 1,4-2,2 % of the population (Diener, 2004). In the treatment protocols NSAIDs and triptans play the greatest role. Recent studies show that Onabotulinum toxin A is a safe, well-tolerated and effective headache prophylactic treatment for CM (Blumenfeld, 2010). The mechanism of action is still not well recognized although it is suggested that toxin inhibits the release of the neuro-transmitters responsible for neural inflammation (Aoki, 2005). Recent studies show encouraging results as the Onabotulinum toxin A seems to be efficient especially in patients who do not tolerate oral preventive drugs (Grazi, 2014).

Strabismus is either intermittent or persistent deviation of ocular alignment. It may have many underlying causes among which abnormal anatomical development of extraocular muscles, impaired neurological input to extraocular muscles, uncorrected refractive error or hereditary factors are the most common ones. There are various treatments associated with strabismus eg. orthoptic exercises and ophthalmic surgery. BTX use in strabismus is modern alternative treatment in this indication. BTX injection temporarily paralyses the extraocular muscle and results in a changed ocular alignment that persists over time (usually after 2-3 months) (Bunting, 2013). Once a muscle is paralysed, opposing muscles take on a greater movement force and the eye position changes allowing the visual axes to move into a straighter eye alignment (Bunting, 2013)

Important is the fact that BTX found also application in the urology field. As it is effective treatment for overactive smooth muscles,

overactive bladder (OAB) reductant for behavioral therapy and oral medications (alfa-blockers, beta-3-agonist) may be treated with it. For many patients with urinary incontinence on the basis of OAB the BTX injection therapy may be the only solution as injected directly to bladder detrusor causes muscle relaxation, weakens the muscle and brings temporary relief (generally up to 6). Side effects may include urinary tract infections and urinary retention that may require catherization. Despite this treatment method is find safe and effective. Also lower urinary tract symptoms (LUTS) associated with benign prostatic hyperplasia (BPH) may be treated in minimally invasive therapy with BTX injections directly to prostate during cystoscopy. This method may be recommended for patients who do not will or cannot undergo surgical procedures (Transurethral resection or enucleation of prostate), due to comorbidity. Intraprostatic injections induces smooth muscle relaxation and atrophy of the prostate gland which result in diminishing LUTS (Chuang, 2006).

Last but not least BTX is used for prevention of facial wrinkles and for excessive sweating therapy in cosmetology and broadly understood anti-aging medicine. Wrinkles are the effect of hyperfunction of muscular action of facial muscles. The BTX mechanism of action makes it an ideal agent in prevention of facial wrinkles (Blitzer, 1997). BTX injections decrease facial muscles activity thus smooth the facial skin and diminishes facial lines. Sweat glands activity is regulated via parasympathetic synapses, thus Ach release may be blocked by BTX, drastically reducing sweat production. Hyperhidrosis results from overactivity of sweats glands based on central dysregulation of autonomic neural system. If topical (stronger antiperspirants, aluminum hydroxide) or systemic (anticholinergics) remedies fail to help, BTX is highly effective therapy (Kreyden, 1997). Injections must be applied to each armpit or hand depending on affected area, resulting in 3-6 months period of time free of the symptoms (Naumann, 1998)

Collagenase C*lostridium histolyticum* (CCH) on the other hand, is an enzyme produced by the bacterium *C. histolyticum* that dismantles collagen. It is used as for the treatment of connective tissue diseases such as Dupuytren's contracture (DC), a condition where the fingers bend towards the palm and cannot be fully straightened, and Peyronie's disease (PD), a connective tissue disorder caused by the growth of fibrous plaques in the soft tissue of the penis.

Dupuytren's disease is nonmalignant fibromatosis of the fibrous skeleton of the hand manifesting in a progressive flexion contracture of the finger. It is more common in developed countries especially among older people (Langer, 2017). Alcohol abuse, diabetes and vibration disease take part in disease etiology. Although surgical methods has been used for many years it was the development of CCH that modernized disease treatment. Surgical treatment is characterized by large number of side effects and undesirable events such as poor healing of the wound (especially in diabetic patients), infections, paresthesia and neurovascular injuries (Denkler, 2010). The effectiveness and safety of CCH use in DC was confirmed in two placebo controlled trials Collagenase Option for the Reduction of Dupuytren's I and II (CORD I and CORD II) (Keller, 2017). Following researches confirmed effectiveness and safety of the procedure with no serious side effects (Rohit, 2019).

As mentioned above PD is a connective tissue disease that affects tunica albuginea of the penis characterized by the plaques deforming penis (because of the penile curvature). Although many treatment options has been described (oral treatment, surgery, Extracorporeal Shock Wave Therapy – ESWT), CCH is the most up to date therapy that represents the most promising advance in the treatment of PD in long time (Jordan, 2014). Effectively used to successfully treat Dupuytren's contracture found an application in PD after a while. Patient are given a series of intralesional injections associated with traction therapy with vacuum devices. Overall effects of the treatment are very good despite the fact that patients with significant curvature may not achieve functionally satisfactory results and may need to undergo surgery (Ralph, 2010).

But Clostridium group bacteria found application also in oncology. Historically it was Vautier who reported in 1813 regression of cancer in patients with gangrene, and in such individuals, the tumour was found to be infected with *Clostridium spp.* (Wei, 2007). Later, it was shown that the causative agent was the bacterium *Clostridium perfringens.* Hypoxia is a pathophysiological feature in the majority of solid tumours. Hypoxic areas in poorly vascularized tumours are main barriers to successful cancer therapy. The blood vessels in tumours are structurally and functionally

abnormal, resulting in unsettled blood supply. The hypoxic microenvironment in solid cancers is ideal for multiplication of anaerobic bacteria. *Clostridium spp*. was shown to cause tumour regression in a rodent model (Roberts, 2014). However, in subsequent clinical studies in human populations, significant therapeutic effect was not demonstrated. Research show also that it is possible to use clostridium for biotechnology purposes in oncology. A recombinant produced C-terminus of the *C. perfringens* enterotoxin was conjugated to gold Nanoparticles to produce a C-CPE-AuNP complex (Becker, 2018). By binding to claudins, the C- CPE should allow to target the AuNPs onto the claudin expressing tumor cells for a subsequent cell killing by application of the gold nanoparticle-mediated laser perforation technique. Furthermore cells without claudin expression (not showing cancer morphology) were spared in treatment. Observations show that C-CPE can be used to functionalize gold nanoparticles in order to specifically and efficiently kill a broad spectrum of claudin expressing tumor cells. The area of oncology treatment using microbiology techniques may require further researches.

The *C. butyricum* strain has been successfully used in the therapy of colorectal cancer (CRC) in the mouse model. Among other, inflammations it is one of the CRC risk factor. Toll Like Receptors (TLRs) are significantly upregulated in intestinal epithelial cells in colitis. The activation of TLRs pathways activates the transcription of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and participates in intestinal tumorogenesis. Chen *et al.* demonstrated that *C. butyricum* can reduce the expression of Toll Like Receptor 4 and NF-κB. In addition, *C. butyricum* can reduce Th17 cell populations, which are vital in carcinogenesis process although its role is not yet understood in spleen. Moreover, the role of *C. butyricum* as an antitumour factor through induction of apoptosis, inhibition of tumour growth and improvement of immune response was described.

Noteworthy is the fact that bacterium is widely used as a probiotic in Asia (particularly in Japan and China), especially during an antibiotic therapy with strong antibiotics (eg III generation cephalosporins or Levofloxacin). Simultaneously prevalence of CRC in Asian countries are much lower than in Western countries (Wong, 2019). Of course etiology of CRC is complex yet in the light of the up to date research, further clinical trials on human model are required.

Clostridium novyi strain NT is bioengineered strain that lacks pathogenic toxins. Due to their affinity to oxygen-depleted parts it can selectively colonize hypoxic regions (eg solid tumors). Labeling *C. novyi* NT with iron oxide found application in modern imaging of solid tumors in Magnetic Resonance Imaging (MRI). *C. novyi* strain NT spores can be successfully labeled with iron oxide nanoparticles for MR imaging in animal model. Noteworthy is the fact that in many animal models intravenous infusion of the spores resulted in tumor size reduction and increased necrosis in histologic preparations. This leaves a chance for the above strain to be used in targeted oncologic therapy based on genetically modified microorganism (GMO) in the future.

Also, the role of *C. difficile* in the wider application of fecal microbiota transplantation (transfer) (FMT) cannot be ignored in the above study. It is the *Clostridioides* infections resistant to conservative treatment that gave impact to the use of this method among others in oncological patients. The oncological patients often have weakened immunity as a result of the specific treatment and the underlying diseases required prolonged antibiotic therapy, which may result in the development of CDI.

DISCUSSION/CONCLUSION

Bacteria of the genus Clostridium have been an important clinical problem for centuries. The eighteenth and nineteenth centuries brought the discovery of etiological factors of the *Clostridium spp.* as pathogens. Even nowadays *Clostridioides difficile* and *C. perfringens* are a real challenge for the health care of many countries. However, over time, we've learned to use *Clostridium spp.* for commercial purposes, including cosmetology and medicine. This mainly applies to the use of botulinum in neurology and anti aging medicine. Also Collagenase *Clostridium histolyticum* (CCH) in the treatment of connective tissue diseases including Peyronie's and Dupuytren's disease therapy. Opportunities for their use in biotechnology and oncological treatment are opening up and the first discoveries often related to animal models encourage further research in this direction.

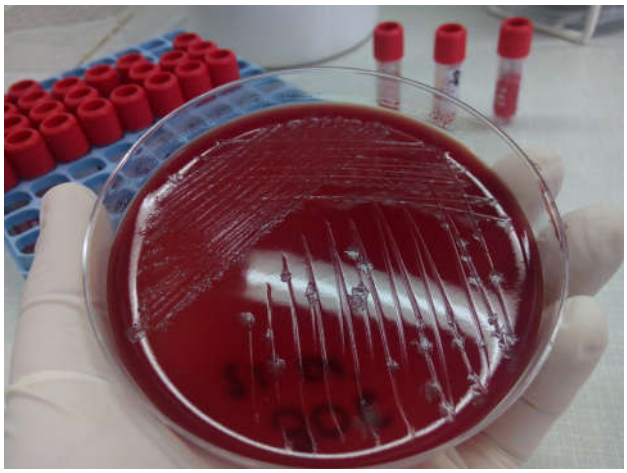

Figure 1. Picture of *Clostridium* sp. colony (photograph taken by M. Kabała)

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