Histological and Ultrastructural Changes of Cardiomyocytes in Experimental Rats with Tail Thrombosis following Subplantar Application of Carrageenin

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**Key Words**
Carrageenin · Rat tail thrombosis · Mitochondrial cardiomyopathy · Granular deposits

**Abstract**

**Objective:** To describe histological and ultrastructural changes of cardiomyocytes in experimental rats following subplantar administration of carrageenin.

**Material and Methods:** In adult rats, an acute inflammatory reaction was induced by subplantar injection of 0.1 ml of 1\% sterile carrageenin solution. In a total of 10 rats, which developed gangrene of tails in 5- to 12-cm-long segments, were killed and their internal organs fixed in 10\% formaldehyde solution and subsequently processed for paraffin embedding. Later, blocks of the ventricular heart tissue were refixed and reprocessed for Araldite embedding and ultrastructure observation. Similarly, the cardiac muscle of control, carrageenin-injected rats which did not develop vascular thrombosis was processed.

**Results:** The cardiomyocytes of rats injected with carrageenin showed focal dystrophic alterations, enlarged mitochondria with densely packed concentrically oriented cristae, and many dense and irregularly shaped deposits with microgranular helicoid organization. Normal cardiomyocytes were observed in control rats. Complicating thrombosis of tail blood vessels leading to extensive tail necroses were also histologically confirmed. **Conclusion:** These findings demonstrate specific pathogenic effect in the cardiovascular system of the carrageenin-treated rats.

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**Introduction**

Carrageenins represent a family of polysaccharide polymers of lectin type extracted from various red seaweeds (\textit{Rhodophyceae}). Biochemically, they are high molecular sulphated polysaccharides – polygalactans [1]. Pharmacologically, they belong to the group of \textit{Mucillagines}, in which alternating units of \(\alpha\)- and \(\beta\)-galactopyranose form linear, strongly anionic polymers with highly flexible molecules of molecular weight >100,000. Some carrageenins (\(\kappa\) and \(\iota\)) at higher concentrations, wind around each other to form double-helical zones. On cooling, the helical formation of the molecules promotes gel formation (mainly \(\kappa\)-carrageenin). On the other hand,
λ-carrageenin, due to its structure of alternating monomeric units of D-galactose 2-sulphate and D-galactose 2,6-disulphate, does not form gel structure. Degradation of natural carrageenin produces low molecular weight poligeenan [2] with different biological properties.

In the food industry, food-grade carrageenin (E407) is used mainly for thickening, suspending and gelling, in preparing cooked meat, sausages and yoghurts. Although the European Commission of Scientific Committee on Food stated that food-grade carrageenin is safe to use [2, 3], some earlier evidence from animal studies has demonstrated that degraded carrageenin, with a molecular weight of ≤30,000, causes ulcerations and malignancies in the gastrointestinal tract [4] and affects incidence of mammary carcinoma [5]. Carrageenin, which can in limited amounts pass through intestinal barrier in experimental animals, may be harmless in healthy organisms, but it may become toxic in groups with disorders of the gastrointestinal tract [1]. The adverse effects of poligeenan (a product of acid hydrolysis in stomach) are ulcerations of intestinal mucosa [6–8], or stimulating neoplastic effect [9]. Ishioka et al. [10] have reported a variety of changes ranging from the initial intestinal inflammations, through ulcerative lesions and squamous metaplasia, to colorectal tumors in experimental rats. Carcinogenesis in the mammary gland was reported by Tobacman [11] and Tobacman and Walters [12] as an effect of poligeenan or λ-carrageenin on mammary myoepithelial cells grown in tissue culture, but Zhou et al. [13] reported an antitumorous effect of λ-carrageenin of various molecular weight.

When administered systemically (parenterally) to experimental animals, carrageenins feature agglutinating activity against blood cells and also against transformed cells in tissue culture [14], mitogenic properties and interaction with some receptors in cell membranes [15]. In general, lectins bind to the saccharide residues of membrane receptors and this reaction can be visualized with a labeled lectin, or it can result in a visible agglutination of cells. Lectins can be used for agglutination of erythrocytes within the range of ABO blood group system, whereas some special lectin fractions show increased hemaglutinating activity [16]. Thomson et al. [17] have shown that intravenous administration of carrageenins to experimental animals developed disseminated coagulopathy. Bekemeier et al. [18, 19] and Hirschelmann and Bekemeier [20] reported thromboses of tail blood vessels in experimental rats after administration of κ-carrageenin. This effect has been later used by Bertelli et al. [21] for testing of antithrombotics.

An effect of carrageenin on the immune system has also been observed, namely a selective toxic effect on antigen-processing macrophages, humoral and cellular immunity [22] and marked suppression of the immune system [23].

In experimental pharmacology research, when testing various anti-inflammatory drugs, the subplantar application of carrageenin solution is widely used (doses of 500–1,000 μg in rat and 300 μg in mouse) to induce aseptic subcutaneous inflammation of paws. Local edematous swelling of the paw can be easily measured, and the anti-inflammatory effect of the investigated drug objectively evaluated [24]. The edematogenic activity of carrageenin decreases with depolymeration, and with the fraction of molecular weight 73,700 and lower this effect is almost absent [25].

Because various adverse effects have been observed in carrageenin-treated rats as outlined above, we decided to investigate histological and ultrastructural changes of carrageenin-induced cardiotoxicity in rats that developed necrotic tails due to thrombosis of tail arteries and veins but also peculiar dystrophic changes in the myocardium.

**Material and Methods**

Female adult Wistar rats (Lab. Animal Farm, Konarovice, Czech Republic) weighing 200–250 g, bred under standard conditions, which developed tail necrosis within 24 h after subplantar injection of 0.1 ml of 1% carrageenin solution in a sterile aqua pro inj. (Carrageenan Type I, commercial grade, Sigma, UK) were studied microscopically. In total, 10 rats out of 20, which developed gangrene of tails in the group of 50 injected experimental animals, were killed the next day under pentobarbital anesthesia. The tails, heart, lung, liver and kidney were removed and fixed in 10% formaldehyde and subsequently processed for paraffin embedding. Similarly, the organs of another 10 injected rats, which did not develop tail necroses, as well as 5 noninjected rats, were processed as controls. Paraffin sections were stained with standard hematoxylin and eosin method, with a modified Giemsa’s method and with a Hale-Mueller’s reaction for visualization of acid mucopolysaccharides, which is based on their binding to the colloidal iron (ferrihydroxidol).

Later, blocks of the ventricular heart tissue were deparaffinized, rehydrated, and refixed with 3% glutaraldehyde for 2 h (SPI, Westchester, Pa., USA) and postfixed with 1% buffered osmium tetroxide (SPI) for 2 h, prior to reprocessing for Araldite embedding (Araldite 502 resin kit, TAAB, Aldermaston, UK). Semithin sections stained with 1% toluidine blue in borax solution were used for light microscopic screening. Ultrathin sections, double-stained with uranyl acetate and lead citrate, were observed under the JEOL 1200EXII (JEOL, Tokyo, Japan) transmission electron microscope at 80 kV.
Results

Necroses of the tail (fig. 1a) with associated vascular thrombosis (fig. 1b) were observed. A positive Hale-Mueller reaction was demonstrable on the surface of erythrocytes in thrombi.

Changes were seen in the left ventricle showing focal dystrophic alteration of cardiomyocytes, with only minimal reactive cellulization in the interstitium (fig. 2a, b). These foci revealed staining irregularities, prominent swelling with hyaline appearance of cardiomyocytes, and in some places also visible granularity of the cytoplasm (fig. 2c). The above-mentioned staining irregularities were impressive in routine paraffin preparations stained with a modified Giemsa’s method (fig. 2a) and in semithin sections stained with Toluidine blue (fig. 2b). These morphological findings permitted provisional diagnosis of carrageenin-induced cardiomyopathy.

In the ultrastructure, longitudinally oriented sections of cardiomyocytes of rats with tail necroses revealed striated myofibrils with signs of rarefaction and disorientation (fig. 3a). Interfibrillar spaces were wide. Prominent, moderately enlarged mitochondria (up to 1.6 µm in length) accumulated near nuclear poles, subsarcolemmal, or were linearly arranged in between the myofibrils. In the interfibrillar locations, the enlarged, mostly spherical mitochondria did not match the length of sarcomeres (fig. 3a). Due to more spherical shapes of mitochondria, the mean area in sections increased from $0.32 \pm 0.09 \mu m^2$ in controls to $0.51 \pm 0.19 \mu m^2$ in carrageen-injected rat. Mitochondrial matrix was always very dense and contained a high accumulation of densely-packed long mitochondrial cristae, which filled up almost completely the profile of this organelle. In some sections, the crowded mitochondrial cristae were arranged concentrically (fig. 3b), while in others, the mitochondria looked...
homogenously dense inside with no resolution of intracisternal spaces.

Some cardiomyocytes also contained, small, dense and irregularly shaped granular-structured deposits located individually or in groups in between the mitochondria, with a tendency of fusing into irregularly shaped units (fig. 3a, c). These granular deposits were neither limited by a membrane unit nor contained any lamellar components inside. In these deposits, the high magnification electron micrographs revealed tightly packed microgranular subunits with helicoid organization (fig. 3c).

Normal cardiomyocytes in the control rats (fig. 4) showed continuous, regularly spaced myofibrils alternating with serially arranged, elongated mitochondria that were closely related to the length of sarcomeres and to transversally cut profiles of the sarcoplasmic reticulum with T tubules. These mitochondria contained transversally oriented cristae within a moderately dense mitochondrial matrix. The only location of grouped or oval-shaped mitochondria were in subsarcolemmal or paranuclear compartments of cardiomyocytes (fig. 4).

**Discussion**

The indicative lesion was the development of necroses in the rat tail caused by vascular thrombosis in a number of experimental rats. An early description of this phenomenon after intravenous or intraperitoneal injection of κ-carrageenin in rats and also in mice was reported earlier by Bekemeier et al. [19, 26] and, in later years, it has been widely used as a model for testing antithrombotics [18, 20, 21].

Tail necroses, due to the thrombosis of the tail blood vessels, in some of the experimental animals were likely
caused by systemic resorption of a small amount of carrageenin injected into the paws, either by direct resorption or mediated transfer through lymphatic circulation. Based on our experience, we assume a great variability in the sensitivity of laboratory rats and mice to the thrombogenic effect of carrageenin, not only according to specific strains, but also due to seasonal variations. This is also supported by findings of Liang et al. [27], as they have been able to facilitate carrageenin-induced tail blood vessel thrombosis in rats (25 mg/kg, i.p.) and mice (150 mg/kg, i.p.) with intravenous application of endotoxin (LPS), which was to act as a sensibilizing agent to the thrombogenic effect of carrageenin.

Myopathic changes in cardiomyocytes, presumed in histological sections, were verified by electron microscopy showing disruption, displacement and rarefaction of myofibils and enlargement and ultrastructural alterations of interfibrillar mitochondria. These ultrastructural features are analogous to some cases of mitochondrial myopathies as described in a variety of muscular disorders where abnormal mitochondria were also found [28, 29]. In myopathies, the mitochondrial changes are manifested by their enlargement up to 14 μm in length (megamitochondria) and marked augmentation of the number and length of mitochondrial cristae which acquire zigzag, concentric or paracrystalline appearance [28, 29]. In our study, the size of mitochondria did not exceed 1.6 μm in length, but most of them lost their regular alignment along the myofibrillar sarcomeres and acquired more spherical shapes with approximately 50% increase in section area. This, together with the impressive increase in number and length of internal cristae and tight packing into concentric formations indicates deranged mitochondrial pattern in myocardial cells with subsequent metabolic abnormalities in rats with systemic reaction to carrageenin. Furthermore, a clear evidence of storage or persistence of the dense granular material was found in the interfibrillar spaces. Since these granular densities are not membrane limited, they were considered as inclusions rather than lysosome-derived structures. By the substructure, as shown in the inset to figure 3c, these granular deposits clearly differ from monoparticulate or aggregated glycogen deposits, which may also be found in the sarcoplasm of normal or pathological cardiomyocytes [29]. These accumulated deposits can also be responsible for pronounced tinctorial abnormalities of cardiomyocytes visualized in histological sections. The chemical nature of this deposited material and the route of its entry into cardiomyocytes remains yet to be elucidated. No ultrastructural signs of endocytosis of such dense material has been observed in the sampled cardiomyocytes. With regard to the high electron density and tight packing, the granular densities in cardiomyocytes differ from the carrageenins experimentally administered and then taken up and stored in macrophages [30, 31, 32] and in some other cells, like mammary myoepithelial cells in tissue cultures [12], and Kupffer cells [33], where they have been observed as a homogenous, fibrillar, or only moderately dense granular material with occasional redundant internal lamellations, located in membrane-limited endosomes.

Interestingly, any sign of terminal damage to cardiomyocytes in terms of apoptosis or necrosis was not observed. This is in contrast to observations of Calkosinski et al. [34] who described apoptotic changes in myocardium of rats in the course of experimentally induced pleurisy, and explained their development with the action of released anti-inflammatory cytokines IL1, ILG and TNF-α. In their experiment, the first myocardial reaction to the remote inflammatory process was an increase in mitochondrial volume within 24 h, followed by derangement of the internal structure – the cristae – within 72 h. Apoptotic nuclei were observed within 120 h of the inflammatory process [34]. No thrombotic complication was reported. We speculate that the apoptotic damage to cardiomyocytes observed in these experiments could be due to intravascularly penetrated carrageenin, as that could have been more easily resorbed from the pleural
cavity within the given time course than from the intraplantar injection in rat paw, as in our experiments. Although, we cannot specify the route of transfer of the injected carrageenin from the connective tissue of the hind paw to the systemic circulation and its uptake by cardiac muscle cells, we would like to point out that care should be taken when using the tissues from these injected animals for other pharmacology examinations even if the tissue is remote from the injection site. Furthermore, the pharmacological data on tested drugs obtained on such experimental models might be modified by alteration of remote organs like the heart. This is also supported by a wide range of hematological changes observed following intraperitoneal injection of carrageenins [35].

Conclusion

This study showed marked mitochondrial lesions in cardiomyocytes in rats following subplantar injection of carrageenin which were found in animals that simultaneously developed thrombus of tail blood vessels with subsequent tail necrosis. In these animals, storage or persistence of a dense granular material was also found in the interfibrillar spaces of cardiomyocytes. These documented structural changes of cardiomyocytes were not described earlier in relation to the thrombogenic effect of carrageenin in rats or other animals. In our opinion, the pathogenic effect of carrageenin in relation to the damage of cardiomyocytes should be given further attention.

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References


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