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The Effect of Intensive Nervous Stimulation on Certain Physico-Chemical Properties of Rat Tail Tendon and Uterus Collagen

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Abstract. The authors simultaneously studied the physico-chemical properties of rat tail tendon (amount of labile hydroxyproline, changes of thermo-isometric tension) and uterine collagen (collagen content, total hydroxyproline, labile hydroxyproline ratio) during the biological ageing of control animals and experimental animals of identical age, exposed to long-lasting stressful stimulation. Similar changes to the tail tendon were found in the uterine collagen. The localisation of increased uterine collagen production under the given experimental conditions, besides the fibroblasts, was partly found in the smooth muscle cells transformed to fibroblastic direction as revealed by electron microscopic investigation of the myometrium.

Key Words
Rat tendon
Rat uterus
Collagen
Isometric tension
Labile hypro
Stress

In our previous studies it was demonstrated that characteristic changes ensue in the hormonal milieu of the organism under the influence of intensive neural stimuli [Árvay, 1964, 1967, 1970]. This statement was verified by adrenal cortical steroid assays, but it was also indicated by the morphological changes reflecting the alterations brought about in the hormonal milieu of the organism.

Results of light and electron microscopic studies, as well as changes of the ^{32}P-isotope storing capacity of anterior pituitary cells unanimously verified that prolonged exposure to overloading nervous stimulation caused an increased function of hypothalamohypophyseal system. The amount of the

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neurosecretory material decreased and a significant 'functional nuclear edema' occurred in the cells of nucleus supraopticus and paraventricularis [ÁRVAY *et al.*, 1960a]. The increased adenohypophyseal hormone production caused by prolonged intensive neural stimuli – an experiment designed by the senior author – resulted in an increased function of the adrenal cortex [ÁRVAY *et al.*, 1960b], thyroid gland [ÁRVAY *et al.*, 1960c] and the ovaries [ÁRVAY *et al.*, 1959], e.g. an intensified activity of the whole neuroendocrine system with all the consequences.

In other experiments we also pointed out that the prolonged overloading nervous stimulation obviously influences the rate of biological ageing in consequence of the characteristically altered hormonal milieu [ÁRVAY *et al.*, 1966; ÁRVAY, 1968]. In these investigations the changed rate of biological ageing was indicated by the physico-chemical changes of the rat tail tendon thermo-isotonic contraction and labile hydroxyproline content.

As a result of these previous experiments, a relationship was found between the effect of intensive neural stimuli and the change of hormonal milieu as well as the change of certain physico-chemical properties of the collagen fibers of rat tail tendon.

In the present experiments we investigated in a simultaneous study, whether a long-lasting stressful stimulation causes a similar change in the rat uterine collagen as in the tail tendon collagen.

Materials and Methods

Female white rats *(R. norvegicus)* were used, kept under constant environmental conditions and on the same diet. The animals were divided into 2 groups at the age of 3 months. After halving all of the litters, one half of the animals were put into the control group, the other half into the experimental group.

The application of intensive nervous stimuli was begun at the age of 3 months and ended at the age of 1 year. During the period of 9 months, a 2-month experimental phase was followed by a 1-month period of repose. The experiment was carried out by means of several stressogen agents. In the isolated rooms of the experimental animals shrill bells were sounded every hour for 5 min (95 phon, 1,000–2,000 Hz high frequency sounds) and flashing reflectors illuminated the animals (1,900–2,000 lux). In addition, every second day the animals were immobilized for 1 h, fixed to a table inclined at 45°, while on the intermediate day they were placed in a shaking apparatus also for 1 h.

Thermo-isometric tension of the rat tail tendon fibers was determined by the thermo-isometric measuring apparatus constructed by TAKÁCS *et al.* [1971] using the method of BROCAS and VERZÁR [1961]. The length and weight of fibers was 5 cm and 2–3 mg, respectively. Number of determinations: 3–5 per animal.

The *'labile hydroxyproline' content* of the rat tail tendon and of the uterus (hydroxyproline eluted in 65°C Ringer solution within 10 min) is given in percent according to MEYER and VERZÁR [1959], the total hydroxyproline content in mg/uterus or in mg/dry material g. The hydroxyproline determinations were performed according to the method of STEGEMANN [1958] modified by WOESSNER [1961] (hydroxyproline mg × 7.46 = collagen amount mg). Two identical parts of the same uterus were used to determine dry weight and hydroxyproline, and a third part for electron microscopic examinations.

For *electron microscopic examinations* the uterus was fixed in a 4-percent Fluka glutaraldehyde (buffered to pH 7.4) for 2 h according to MILLONIG [1961], then a subsequent fixation was performed for 1 h in 1% osmiumtetroxide. Tissue blocks were dehydrated in ethanol series and then embedded in Durcupan ACM epoxy resin. Sections were prepared with LKB ultramicrotome, mounted on copper grids and stained with lead citrate according to REYNOLDS [1963]. The sections were examined by means of a Zeiss 2D Elmiskop type electron microscope. The micrographs were taken on Gevaert plates at 50 kV accelerating voltage and with electronic magnification of 2,000–12,000×. Photooptical enlargements were made if needed.

Results

The results of the first series of our experiments are summarized in table I. The experiments were begun when the animals were 3 months old and the complex intensive neural stimuli were applied from this period according to our specially designed procedure. The animals were sacrificed and the examination of the uterus took place at the age of 3, 4, 6, 9 and 12 months immediately after a 30, 90, 150 and 200-day period of neural traumatization. Control animals of identical age were killed at the same time and their uterus and tail tendon fibers were examinated also. The following is evident from the data in table I.

1. *Total hydroxyproline content* of the control uteruses increases with age. In the case of the 3-month-old animals the mean 1.83 ± 0.16 mg total hydroxyproline increases to 6.16 ± 0.30 mg at the end of 1 year.

2. Mean values of the total hydroxyproline content of the uteruses from the experimental animals at the age of 3 months are higher after 10 days of traumatization than those of the control group, 1.91 ± 0.18 versus 1.83 ± 0.16 mg, but the difference is not significant. From the age of 4 months, however, the total hydroxyproline content of the uterus of experimental animals is significantly higher, compared to the controls. After 30 days of traumatization the values are 3.02 ± 0.13 mg compared with the 2.32 ± 0.11 mg of the control uteruses after 200 days of traumatization – at the age of 1 year – the values increase to 8.21 ± 0.34 mg as compared with the 6.16 ± 0.30 mg of controls.

Table I. Changes of collagen content in the uterus under the effect of prolonged nervous stimulation overload

Age group	Age, months	Number of animals	Weight of animals, g	Duration of the nervous stimulation, days	Wet weight of uterus, mg	P	Dry weight of uterus, mg	P	Hydroxyproline total, mg/uterus	P	Hydroxyproline concentration, mg/100 mg dry tissue	Hydroxyproline percent 'labile' at 65°C	P
I	3	6	200±17	control	382±35	>0.05	15.9±2.31	>0.05	1.83±0.16	>0.05	3.3509	9.4±2.61	>0.05
	3	6	212±11	10	370±36		15.2±2.42		1.91±0.18		3.3962	9.5±2.50	
II	4	6	250±13	control	390±40	<0.001	16.8±2.38	<0.05	2.32±0.11	<0.01	3.5409	7.5±1.12	<0.05
	4	6	206±18	30	503±31		18.2±2.41		3.02±0.13		3.2988	6.4±1.08	
III	6	6	243±12	control	436±28	<0.001	18.4±2.30	<0.01	2.81±0.14	<0.05	3.5262	6.1±0.08	<0.05
	6	6	213±19	90	510±38		21.6±2.51		3.63±0.20		3.2952	5.1±0.86	
IV	9	6	231±16	control	472±41	<0.05	19.3±2.41	<0.01	4.25±0.21	<0.01	4.6654	5.4±0.76	<0.05
	9	6	215±10	150	536±39		21.6±2.18		5.63±0.23		4.8628	4.2±0.67	
V	12	8	265±18	control	521±60	<0.05	19.6±2.42	<0.01	6.16±0.30	<0.001	6.0324	4.9±0.34	<0.01
	12	8	245±20	200	613±51		21.8±2.43		8.21±0.34		6.1436	2.8±0.24	

3. The *labile hydroxyproline content* of the uterus, i.e. the amount of hydroxyproline eluted from a 65 °C Ringer solution within 10 min decreases with age: at the age of 3 months (controls) it was 9.4 ± 2.61 %, that of the 1-year-old animals decreased to one half of this: 4.9 ± 0.34 %.

4. The effect of a 10-day' traumatization is not yet reflected in the percent amount of labile hydroxyproline. After a 30-day application of intensive neural stimuli, however, the labile hydroxyproline content is only 6.4 ± 1.08 as compared with the 7.5 ± 1.12 % of the control. This difference becomes even greater later and at the age of 1 year – after a 200 days neural traumatization – the labile hydroxyproline content of the uterus was 2.8 ± 0.24 % while that of the controls was 4.9 ± 0.3 %.

5. Both the wet and dry weight of the 'experimental' uteruses is greater compared to that of controls.

Ultrastructural Observations on the 1-Year-Old Animals

In the uterus of the control rats regular smooth muscle cells could be seen in the muscular layer. The distribution of the chromatin in the nuclei was uniform. Myofilaments filled the cytoplasm, cell organoids could be seen only rarely. A scanty amount of glycogen granules and pynocytotic vesicles completed the ultrastructural picture of the smooth muscle, as is well known in the literature [GANSLER, 1960, 1961]. The intercellular space is relatively narrow, very few collagen fibers can be observed in it. The smooth muscle cells arrange themselves in regular bundles, among them other cell types (fibroblasts) can be seen only exceptionally (fig. 1 and 2).

In the smooth muscle cells of the uterus of the experimental animals the chromatin of the nucleus is coarser, it concentrates chiefly along the nuclear membrane. In the cytoplasma, groups of ribosomes (polysomes) appear among the myofilaments. Numerous mitochondria and very well developed Golgi apparatus can be seen. Near the surface of the cell vesicles appear containing electron-dense material of medium degree. The intercellular space is significantly dilated in consequence of the marked collagen proliferation, so that the cells – shifted away from one another – are situated dissociatedly.

Experimental changes are demonstrated in representative micrographs (fig. 3 and 4).

In these experiments it was investigated whether the thermo-isometric tension and the labile hydroxyproline content of the tail tendons of experimental animals change under the influence of complex stressfull stimulation. Although we already have demonstrated that intensive neural stimuli

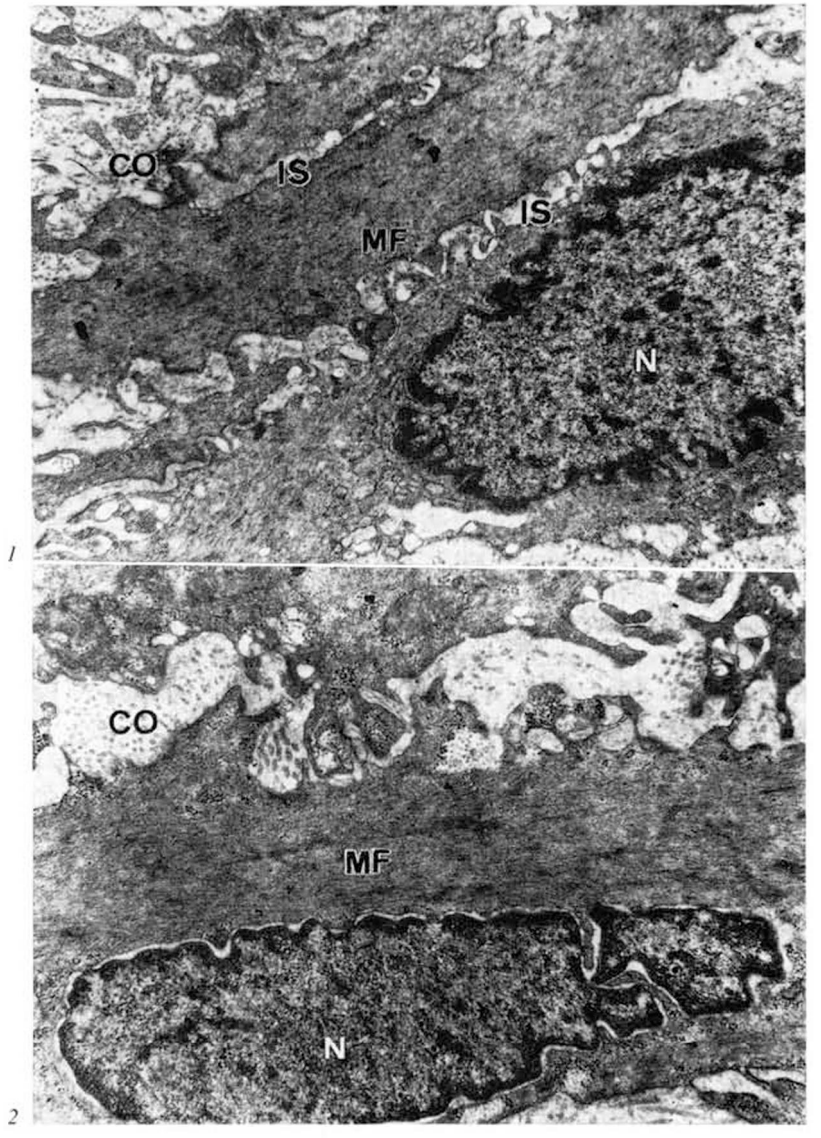
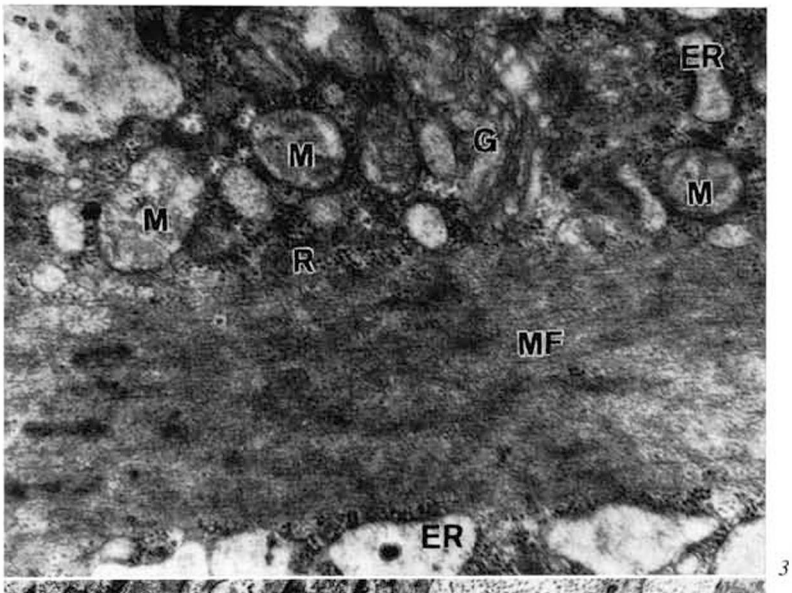


Fig. 1 and 2. One-year-old control animal. Myometrial smooth muscle cells. The intercellular space (IS) is narrow and contains collagen fibers (CO) in small amounts. The cells are densly packed with myofilaments (MF). Cell organoids are practically absent. Nuclear chromatin (N) pattern is regular. × 9,000 and × 10,000.



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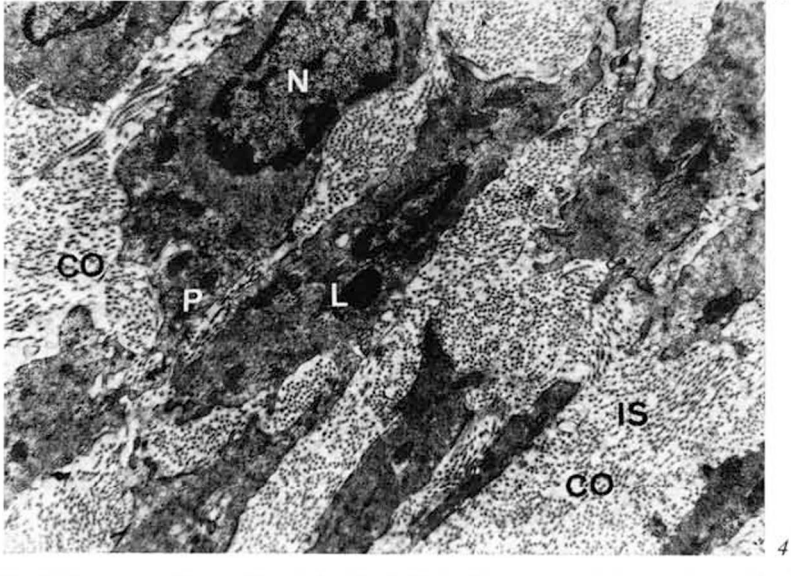

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Fig. 3. One-year-old experimental animal. Part of a myometrial smooth muscle cell showing myofilaments, mitochondria (M), a well-developed Golgi-area (G) and dilated cysternae of rough surfaced endoplasmic reticulum (ER). RNA particles (R) are also present. × 15,000.

Fig. 4. Survey picture from the uterine myometrial layer of a one-year-old experimental animal. The intercellular space is dilated and filled with collagen fibers. Thus, the cells are dissociated. The chromatin show concentration beneath the nuclear membrane. Lysosome-like bodies (L) and pinocytotic vesicles (P) are also present. × 38,000.

influence the thermo-isotonic but not the thermo-isometric contractions of rat tail tendon fibers, significant difference was registered only in the 12th and 24th month respectively.

Figure 5 demonstrates that the 10-day traumatization applied at the age of 3 months does not influence the thermo-isometric tension of the fibers significantly. In the other age-groups, however, upon a further application of the intensive neural stimulation, the thermo-isometric tension increases at a greater extent than in the control animals. Thus, at the age of 12 months, the thermo-isometric tension exceeds the control value by almost 2 g. According to this experimental series the thermo-isometric tension of the tail tendon fibers increases parallel with the progress of ageing. We were able to ascertain also that intensive neural stimuli increase this process and that a 30-day traumatization results in a marked difference as compared to the values of the control animals.

Figure 6 represents the results of the labile hydroxyproline determination performed on the collagen fibers of rat tail. It can be seen that both in the

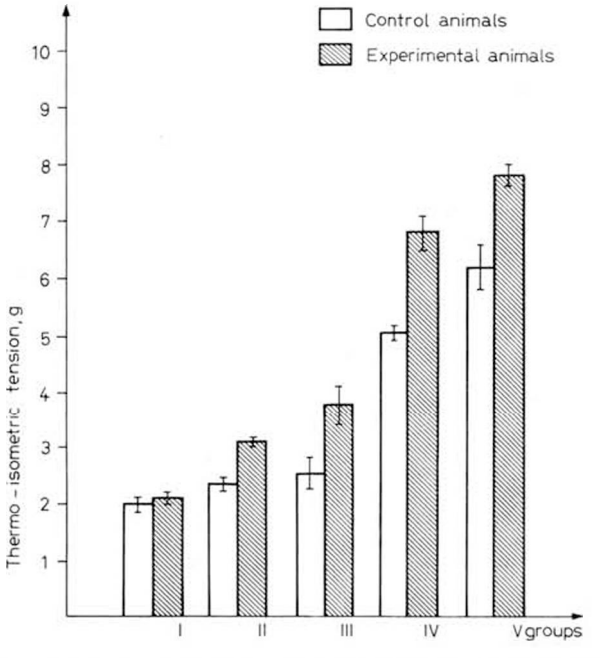

Fig. 5. Changes in the rat tail tendon thermoisometric tension at the age of 3, 4, 6, 9 and 12 months (I, II, III, IV and V groups).

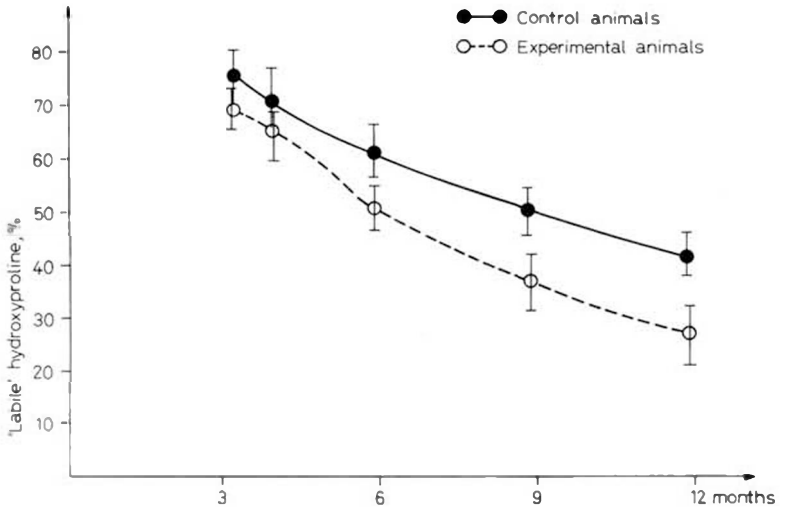

Fig. 6. Influence of stressful stimulation on the 'labile' hydroxyproline ratio in the tail tendon of ageing rats.

control and in the experimental animals decreasing values can be found from the age of 3 months to the age of 12 months, i.e. to the end of our experiments. This decrease was greater in the experimental animals than in the controls. From the age of 6 months, i.e. after a 90-day traumatization the difference is already significant.

According to our establishments too, the labile hydroxyproline percent in the collagen fibers of rat tail decreases during life: at the age of 1 year it falls to almost a half of the value determined at the age of 3 months. This decrease becomes even more marked under the influence of intensive neural stimuli. Hence, we may ascertain that our special, complex intensive neural stimulation influences the percent amount of the labile hydroxyproline in the tail tendon fibers by increasing its physiological decline associated with biological ageing.

Discussion

According to our experimental results the collagen content of the uterus changes with age, the total hydroxyproline content increases and the percent of the labile hydroxyproline decreases. A similar change associated with age was found in the tail tendon fibers too. Our results regarding the

changes of the collagen content of the uterus in connection with age are in complete agreement with the data of SCHAUB [1964/65]. Similar changes were verified by VERZÁR and MEYER [1961] in tendons, by VERZÁR [1960] in the skin, by SCHAUB [1963a] in striated muscles and in the myocardium [1964], and in the parenchymal organs [1963b].

In order to attain to a more complete understanding of the essence of these processes, in previous experiments [ÁRVAY *et al.*, 1963; ÁRVAY and TAKÁCS, 1963, 1964/1965, 1965, 1966] we investigated the effect of the changes of the hormone-milieu, gravidity, puerperium, castration, and the application of intensive neural stimuli on certain physico-chemical properties of the rat tail tendon. Dealing with the mode of action, it was found in these experiments that an important role was played by the oestrogens and adrenal cortical steroids in bringing about in the collagen changes characteristic of biological ageing.

In the present experiments the effect of intensive neural stimuli was investigated partly on the physico-chemical properties of the rat uterine collagen and partly – as a supplement of our previous experiments [ÁRVAY *et al.*, 1966] on that of the rat tail tendon collagen. In the course of the continuous application of intensive neural stimuli the collagen content and the degree of thermo-isometric tension of the tail tendon is significantly greater in the experimental animals than in the controls of identical age. The long-lasting intensive stress, therefore, produced changes of such a degree and character which exceeded the physiological changes characteristic of and accompanying biological ageing.

According to our previous investigations, multiplex stressogen agents ensued a far-reaching structural and functional change of the endocrine system: a marked increase of function [ÁRVAY, 1967]. In addition to the evidence mentioned above, the electron microscopic examination of the adenohypophysis and its target organs also documented the ultrastructural proofs of this function increase [ÁRVAY *et al.*, 1971]. Thus, we are justified in emphasizing the significance of the altered hormonal milieu in the manifested changes brought about by the effect of the intensive neural stimuli and indicated by the physico-chemical changes in the uterus and rat tail, as we have already verified in the course of biological ageing.

During our present experiments the electron microscopic examination of the uterus verified an undoubted increase of collagen production in the uterus of the rats exposed to the effect of intensive neural stimuli. As can be seen, the electron microscopic pictures are also in agreement with our biochemical results.

The increased *fibroblastic* activity in the smooth-muscle cells of the uterus – brought about presumably by estrogen stimuli – may be held responsible for the increased collagen production in the uterus. Such a *fibroblastic transformation* of the smooth muscle cells is not uncommon [Ross and Klebanoff, 1967; Ladányi and Lelkes, 1968; Weiss, 1968]. The fibroblastic activity of the smooth muscle cells cannot be disputed, because the collagen fiber proliferation is associated topographically with the smooth muscle cells and at the same time the ultrastructural signs of the synthesis and excretion of the extracellular substance (protein and mucopolysaccharides) can be observed in these cells. Naturally, the significance of the fibroblasts cannot be ignored either. Their activity was observed primarily in the interstitial septa.

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