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PYRAZINAMIDE-MEDIATED CHANGES IN RAT TYPE I COLLAGEN AND SPERMATOGENESIS INDICES

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Abstract: Necessity of tuberculosis chemotherapy adverse effects minimization requires a comprehensive evaluation of the effects of antitubercular drugs on reproductive system and extracellular matrix proteins. Wistar albino male rats (160–200 g) were divided into three groups: I – received pyrazinamide *per os* at a dose of 1000 mg/kg bw/day, II – at a dose of 2000 mg/kg bw/day, in both group it was given for 60 days; III – intact animals. The contents of amino acids in rat type I collagens were determined using an amino acid analyzer. Morphological analyses were carried out by an optical microscope. The study of the effects of pyrazinamide administered in different doses on type I collagen amino acid contents, testis cells morphologic and morphometric parameters and spermatogenesis demonstrated the presence of pyrazinamide-mediated quantitative and qualitative changes in male rat reproductive organs, spermatogenic epithelial cells and extracellular matrix proteins in comparison with norm.The largest number of changes were established at a dose 2000 mg/kg b.w./day. The observed collagen molecules changes could hence affect the properties and correct functioning of spermatogenic epithelium and other tissues of reproductive organs. They might be caused by pyrazinamide *via* cytochrome P450 2E1 induction, reactive oxygen species production or direct action of this compound on protein biosynthesis processes.

Keywords: pyrazinamide, spermatogenesis, type I collagen

One third of the world's population has positive tuberculine tests and this number is increasing (1). Situation became more serious as direct action of *Mycobacterium tuberculosis* is aggravated by wide spectra of accessory pathologies accompanying tuberculosis.

Connective tissue disturbances and especially qualitative changes of collagen synthesis always accompany these pathologic processes. For example, fibrosis is one of the major causes of post-treatment morbidity in tuberculosis. Among this, tuberculosis should be considered as a possible cause of cutaneous leukocytoclastic vasculitis (a small-vessel vasculitis localized to the skin) (2). The molecular basis of fibrosis in active and healed tuberculous lesions as other tuberculosis-caused collagen pathologies is yet to be fully characterized (3, 4). The current therapeutic approaches for such processes, which are characterized by fibroblast proliferation and extracellular matrix remodeling, are unsatisfactory (5).

Among tuberculosis itself, simultaneous and long-term usage of multiple drug combinations also often causes various negative effects on connective tissues collagens (6) as most of these compounds have their profound effects on amino acids and proteins metabolism. Previously, such effects were demonstrated for pyrazinamide, one of the most popular antitubercular preparation (7).

Taking into account that quantitative changes of collagens structures strictly correlated with such parameters of male reproductive system as % of normal sperm and sensitivity to testicular degeneration (8), it becomes obvious how important is thoughtful analysis of pyrazinamide simultaneous effects on collagen and reproductive system for further search for measures to lower its complex toxicity and increase its efficiency.

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The aim of the study was to estimate putative changes in rat type I collagen amino acid contents, testis cells morphologic and morphometric parameters and spermatogenesis with usage of different doses of pyrazinamide.

EXPERIMENTAL

Wistar albino male rats, body weight (b.w.) of 160–200 g, were used in the study. Animals were kept under standard conditions of nutrition, water and light regimes.

The study was carried out according to national and international guidelines and the law on animal protection was observed. All animal studies were performed in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and approved by the Institutional Animal Care and Use Committee.

In the experiments, pyrazinamide (pyrazine-2-carboxyl amide) was used in tablets (500 mg of active compound in each) produced by the Borzhagovsky Chemical and Pharmaceutical Enterprise, Ukraine (series P.10.00/02271). Rats were kept in quarantine for 10 days, then they were randomized into experimental and control groups. Each group included 6 animals. Pyrazinamide suspended in 1% starch gel was given by gavage at a dose of 1000 mg/kg b.w./day (group I) and 2000 mg/kg b.w./day (group II) for 60 days (duration of spermatogenesis process and time of germ cells maturation in epididymis). The control group received only starch gel in corresponding volumes. Choice of doses was based on the previous data available in NIEHS Technical Report on the Reproductive, Developmental, and general Toxicity Studies of Pyrazinamide (http://ntp.niehs.nih.gov/files/AIDSTox011.pdf) (8). Body weight gain, activity, behavior and appearance of the rats were observed throughout the period of pyrazinamide administration.

After 60 days of the experiment, rats of the experimental (groups I and II) and control groups were sacrificed *via* cervical dislocation under mild diethyl ether narcosis. The rats' skin and testis were used for biochemical assays.

Skin type I collagens were extracted and purified according to (9). All procedures were carried out in cold regime (+4°C). Fractionation of pure type I collagens was carried out with NaCl according to (9, 10). Collagen preparations purity was controlled electrophoretically (11). Collagen fractions were hydrolyzed – 24 h, 6 M HCl, 105°C (12). Their amino acid compositions were analyzed by ion exchange chromatography on the amino acid analyzer AAA-881 (Czech Republic).

For investigation of morphologic and morphometric parameters of germ cells and spermatogenesis processes, right testicle was used. Its radiuses, volume and weight was measured. Then, it was fixed in 10% solution of neutral formalin, dehydrated in ethanol solutions and embedded in paraffin. Histologic sections (6 mm) were stained by hematoxilin and eosin. Microscopic studies were carried out with microscope Cytophan (Leica Microsystems Wetzlar GmbH). Determination of spermatogenesis index in testicles was carried out according to four points system (13). Simultaneously with determination of above mentioned quantitative parameters of spermatogenesis, also qualitative changes of spermatogenetic epithelium: cells desquamation, epithelium exfoliation from tubule basal membrane and presence of cell-free regions ("windows") were investigated.

The obtained data were calculated by one-way analysis of variance (ANOVA). Data were compared using Tukey test. Differences were considered to be statistically significant at $p < 0.05$.

RESULTS

Everyday observation over animals during the whole period of pyrazinamide introduction testified the absence of any changes in their state: appearance, behavior, activities, hair; water and feed consumption of experimental animals didn't differ from those of the control group. No animal died during the period of experiment.

Testicles external examination at rats autopsy did not mark any visible pathology: organs were without any traces of edema, with normal blood filling.

Another situation was with morphometric indices. Morphometric parameters of rat male gonads and some indices of spermatogenesis with pyrazinamide treatment are presented in Table 1.

Lowering of testicle medium volume to 23% in group of animals treated with 2000 mg/kg of pyrazinamide and dose-dependent decrease of germ cells production (in group of animals treated with 1000 mg/kg of pyrazinamide – to 18% and with 2000 mg/kg of pyrazinamide – to 32%) were detected.

Morphometric changes in reproductive organs were accompanied with changes in spermatogenic epithelium. Results of rat testis spermatogenic epithelium microscopy are presented in Table 2.

According to these data, the index of spermatogenesis did not greatly changed in experimen-

Table 1. Some indices of rat testis with pyrazinamide administration during whole period of spermatogenesis (Ě ± SE, n = 6).

Indices	Animal groups		
	Control	Pyrazinamide, 1000 mg/kg	Pyrazinamide, 2000 mg/kg
Testis weight (g)	1.79 ± 0.03	1.7 ± 0.04	1.58 ± 0.24
Testis volume (mm³)	1838.7 ± 97.4	1770.7 ± 56.3	1419.9 ± 97.3* #
Number of spermatozoids (10^6/mL)	37.6 ± 1.32	30.7 ± 1.4*	25.7 ± 0.43* #
Pathologic forms of spermatozoids (%)	Folded, isolated	Folded, isolated	Folded, isolated

* $p < 0.05$ statistically significant in comparison with control; # $p < 0.05$ statistically significant pyrazinamide, 1000 mg/kg group *vs.* pyrazinamide, 2000 mg/kg group

Table 2. Rat spermatogenetic epithelium morphometric indices with pyrazinamide administration during whole period of spermatogenesis (M ± m, n = 6).

Indices	Animal groups		
	Control	pyrazinamide, 1000 mg/kg	pyrazinamide, 2000 mg/kg
Spermatogenesis index	3.6 ± 0.01	3.76 ± 0.02	3.64 ± 0.01 #
Number of spermatogonia	71.4 ± 1.32	74.7 ± 1.32	42.8 ± 7.8* #
12-th stage of meiosis, %	2.3 ± 0.32	5.57 ± 0.7*	5.7 ± 0.7*
Desquamated epithelium	7.3 ± 1.2	9.6 ± 2.25	13.2±1.14*
Exfoliation of epithelium, %	0.5 ± 0.28	0.86 ± 0.5	3.8 ± 1.4*
"Windows",%	–	–	3.0± 1.5*

* $p < 0.05$ statistically significant in comparison with control; # $p < 0.05$ statistically significant pyrazinamide, 1000 mg/kg group *vs.* pyrazinamide, 2000 mg/kg group

tal groups and was on the mean level of physiological norm (14) for rats. At the same time, a number of spermatogonia in the group of animals treated with 2000 mg/kg of pyrazinamide was greatly decreased in comparison with other groups. In the experimental groups there were increased levels of cells with 12-th stage of meiosis.

Among above mentioned quantitative changes, qualitative changes of spermatogenetic epithelium were also present in convoluted seminiferous tubules. Desquamation of epithelial cells was observed in group of animals treated with 2000 mg/kg of pyrazinamide (Fig. 1). Great degenerative changes such as epithelium exfoliation from tubule basal membrane and the presence of cell-free regions ("windows") also were especially characteristic for this experimental group (Figs. 2, 3).

Pyrazinamide effects on spermatogenic epithelial cells was accompanied by structure changes in extracellular matrix (especially in connective tissue proteins). The changes in rat type I collagens amino acid contents induced by pyrazinamide were profound as compared to control (Table 3).

Statistically significant changes were registered in type I collagen with pyrazinamide administration at dose 1000 mg/kg for 9 amino acids and at dose 2000 mg/kg – for 10 amino acids.

Type I collagen of rats with pyrazinamide at dose 1000 mg/kg contained lower contents of proline (-19.8 %), glycine (-7,2%) and alanine (-23,2%) simultaneously with higher contents of lysine (+23,0%), arginine (+26,9%), aspartic acid (+21,0%), glutamic acid (+22,0%), tyrosine (+37,8%) and phenylalanine (+54,9%). This protein from rats with pyrazinamide at a dose of 2000 mg/kg contains lower contented of proline (–16,2%), glycine (–15,3%) and alanine (–22,0%) simultaneously with higher contents of lysine
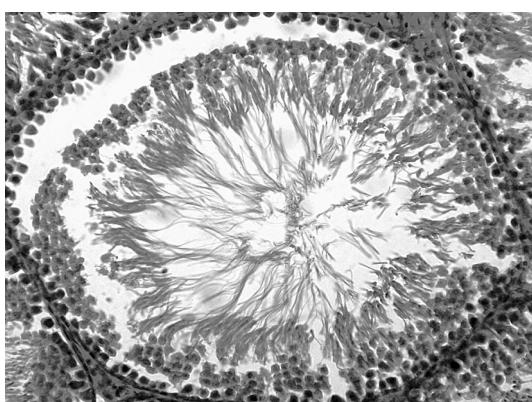
Figure 1. Desquamation of epithelial cells, observed in group of animals treated with 2000 mg/kg of pyrazinamide during whole period of spermatogenesis. Hematoxilin and eosin, 400×
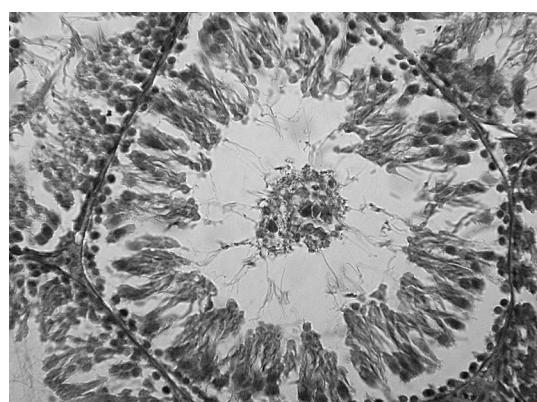

Figure 3. Epithelium exfoliation from tubule basal membrane, observed in group of animals treated with 2000 mg/kg of pyrazinamide during whole period of spermatogenesis. Hematoxilin and eosin, 400×
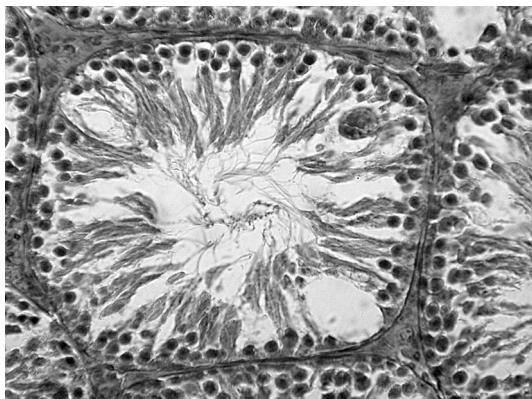
Figure 2. Presence of cell-free regions ("windows"), observed in group of animals treated with 2000 mg/kg of pyrazinamide during whole period of spermatogenesis. Hematoxilin and eosin, 400×

(+22,5%), histidine (+79,2%), arginine (+28,0%), threonine (+46,6%), glutamic acid (+22,0%), isoleucine (+42,2%) and phenylalanine (+68,0%). For the majority of amino acids contents pyrazinamide effects were dose-dependent.

DISCUSSION

Observed pyrazinamide-mediated changes in rat reproductive system could be a result of compensatory events in testicles (Tables 1, 2). An increased level of first order spermatocytes meiotic division accompanied by a decreased production of spermatogonia had to provide further development of germ cells in physiologically necessary quantity. However, this did not happen and spermatozoon production at such conditions of xenobiotics intro-

duction remained decreased in comparison with control group (Table 2).

In the case of spermatogenic epithelium, pyrazinamide effects obviously could not be fully compensated and detected changes could be a result of degenerative processes in rat reproductive organs.

An important aspect of spermatogenesis involves the detachment of germ cells from the basement membrane and their subsequent migration towards the tubule lumen. Procollagen I, a precursor of type I collagen, is a trimer consisting of two α1 chains and one α2 chain, whose sequences are encoded by two different genes, namely Col 1a1 and Col 1a2, respectively (15). Distribution of procollagen I within the seminiferous tubules of immature and adult mice correlates with the process of germ cells attachment to and detachment from the basement membrane. The unique distribution pattern of procollagen I in adult mouse testis implies possible roles of Col 1a1, Col 1a2 and procollagen I in regulating the adhesion of spermatogonia and preleptotene spermatocytes to the basement membrane and the detachment and migration of later spermatocytes and spermatids towards the lumen during spermatogenesis (16).

The profound pyrazinamide-mediated changes in collagens amino acid compositions found (Table 3) presumably brought to disturbances in proteins molecules physicochemical behavior. Decreased levels of proline inevitably affected collagen helix properties (mechanical strength, elasticity/rigidity) as in norm its structure presumed to consist from triplets Gly-X-Pro or Gly-X-Hyp (17).

The hydroxylysine residues along with lysine and histidine (17) participated in collagen cross-linking. Changes in ratio hydroxylysine : lysine :

Table 3. Rat skin type I collagen amino acid contents in control and with pyrazinamide administration (M ± m, n = 5, residues/ 1000 residues)

Amino acid	Control (norm)	Pyrazinamide 1000 mg/kg	Pyrazinamide 2000 mg/kg
Hydroxylysine	4.30 ± 0.10	3.40 ± 0.30	4.50 ± 0.60
Lysine	29.80 ± 1.30	36.70 ± 0.90*	36.50 ± 3.00*
Histidine	4.80 ± 0.20	5.80 ± 0.90	8.60 ± 0.90*
Arginine	50.20 ± 0.60	63.70 ± 4.30*	64.30 ± 3.70*
Hydroxyproline	92.70 ± 2.30	93.70 ± 3.00	101.90 ± 6.30
Aspartic acid	44.70 ± 1.10	54.10 ± 1.80*	47.80 ± 2.00 [#]
Threonine	17.80 ± 0.70	16.20 ± 3.60	26.10 ± 2.70*
Serine	35.80 ± 1.00	43.80 ± 3.20	39.30 ± 3.40
Glutamic acid	75.20 ± 1.50	91.80 ± 2.20*	91.80 ± 2.30*
Proline	130.60 ± 3.00	104.70 ± 3.40*	109.50 ± 6.60*
Glycine	324.40 ± 3.70	301.60 ± 9.60*	274.70 ± 18.40*
Alanine	105.20 ± 2.10	80.70 ± 6.50*	82.00 ± 7.00*
Valine	26.40 ± 0.60	24.00 ± 2.30	30.10 ± 1.90
Methionine	6.80 ± 0.10	6.30 ± 0.50	9.10 ± 1.90
Isoleucine	12.80 ± 0.80	15.30 ± 1.60	18.20 ± 1.50*
Leucine	29.20 ± 1.70	31.40 ± 0.40	30.30 ± 0.50
Tyrosine	3.70 ± 0.20	5.10 ± 0.40*	4.80 ± 0.70
Phenylalanine	12.20 ± 0.50	18.90 ± 0.60*	20.50 ± 1.80*

* $p < 0.05$ statistically significant in comparison with control; [#] $p < 0.05$ statistically significant pyrazinamide, 1000 mg/kg group *vs.* pyrazinamide, 2000 mg/kg group

histidine residues could seriously influence the number and type of cross-links in collagen fibrils (thus on mechanical strength, elasticity/rigidity of extracellular matrix).

Changes in number of arginine, aspartic acid, threonine, serine and glutamic acid residues could cause great changes in surface charge of collagen molecule (17), whereas changes in quantity of isoleucine, tyrosine and phenylalanine residues could influence the level of collagen helix rigidity (17).

The changes in arginine, glycine and aspartic acid residues could affect the number of domains Arg-Gly-Asp, which are responsible for processes of cells adhesion on collagen structures (18–21). Arginine and glycine residues in collagen molecule also are parts of special locuses responsible for interactions with chaperons and for procollagen to collagen processing (22).

Thus, the presence of pyrazinamide-mediated quantitative and qualitative changes in male rat reproductive organs, spermatogenic epithelial cells and extracellular matrix proteins (type I collagens) was demonstrated in comparison with norm. With

pyrazinamide administration, possibly type I collagen molecules could be formed with changed helix structure, surface charge, rigidity, number and types of cross-links and specific locuses responsible for cell adhesion, interaction with chaperons and procollagen processing to collagen. Such collagen molecules changes could hence affect the properties and correct functioning of spermatogenic epithelium and other tissues of reproductive organs.

It could be supposed that such changes could be caused by pyrazinamide derivatives formed *via* cytochrome P450 2E1 induction (23) as it was previously demonstrated for pyrazole (24). The cytochrome P450 2E1-derived reactive oxygen species mediated paracrine stimulation of type I collagen synthesis on different stages of this process (24) and caused genotoxic effects on rodents germ cells (25).

Besides of such cytochrome P450 2E1-mediated pyrazinamide influence on collagen synthesis, pathologic changes in amino acid metabolism (caused by this compound) in whole organism (7) could also affect on collagen metabolism and structure. This supposition are in good accordance with

results of other authors. The majority of amino acids *in vitro* can regulate protein biosynthesis at the level of translation *via* stimulation of 70Kd-ribosomal protein S6-kinase (26). Another investigations demonstrated amino acids suitability to control protein synthesis in cells through both translational and pretranslational mechanisms (27). Present pyrazinamide-mediated changes in collagen amino acid compositions and free amino acid contents (7) had analogous characters for free lysine, histidine, arginine, threonine, serine, isoleucine, tyrosine and corresponding amino acid residues in collagens.

On the other hand, taking into account collagen genes polymorphism not only with pathologies but also in norm (28, 29), the detected changes could be a result of pyrazinamide derivatives- and reactive oxygen species-mediated disturbances in rates of transcription of different genes from the same collagen type I superfamily (as it was previously demonstrated for osteogenesis imperfections) (30).

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