HISTOLOGICAL PROPERTIES OF THE ISCHIADIC NERVE IN RATS INTOXICATED BY ORGANIC SOLVENTS

Ljiljana Blagojević1, Jelena Blagojević2, Slobodan Dožič3, Slavko Ćušić4

1Institute of Occupational Health, Niš, Serbia and Montenegro
2Faculty of Medicine, Florence, Italy
3KCS – Institute of Pathology, Faculty of Medicine, Belgrade, Serbia and Montenegro
4Military Medical Academy, Belgrade, Serbia and Montenegro

Summary. The incidence of neurophysiological disorders and polyneuropathies has been seen as a consequence of long-term occupational exposure to organic solvents. The aim of the study was to determine, and histologically quantify the changes in the sciatic nerves of the rats intoxicated by inhalation of organic solvents (toluene, xylene, n-butylacetate, acetone and white-spirit) in the concentrations of 3,000 ppm (6 hours a day, 5 days a week, except for week end during the period of 8 weeks).

Initial changes, indicating toxic neuropathy, i.e. axonal degeneration, accompanied by accumulation of soft membrane neurofilaments, as well as of mitochondria, were perceived. This finding is based on the appearance of endoneural edema, more pronounced in the central parts of the bundle and subperineurally (light microscopy), while individual fibres (teased preparation) showed a focal, oval or round, tomaculum-like body myelin swellings, with compression on axons and sporadic degeneration. Electro-microscopy showed clear degenerative changes of myelin in the form of "tomaculous" reduplication of myelin lamellae, sometimes multiple within the whole circumference with compression on very atrophic axons and coagulation of organellae (neurofilaments and neurotubules, proliferated endoplasmic reticulum), as well as with mitochondria accumulation in the Schwann cell cytoplasm in the paranodal region.

Key words: N. ischiadicus, axonal degeneration, organic solvents, rats

Introduction

Neuropsychiatric disorders are among the most common diseases of workers with long term occupational exposure to organic solvents (OS) (1,5, 9,12). The most common consequences of this exposure to a mixture of OS (toluene, xylene, white-spirit, n-butylacetate and acetone) include an impairment of peripheral nerves, clinically diagnosed as toxic polyneuropathies (3,10,11). Electroneurographic measurements confirm subclinical toxic neuropathies, which appear earlier and more frequently than the clinically manifested ones. Pathohistological disorders are recognized before neurophysiological deviations and they represent the most sensitive indicator in early detection and determination of the morphological substrate of peripheral nerve impairment. Neurotoxic effects were provoked in Wistar rats by a controlled exposure, while the axonal degeneration was pathohistologically quantified by light microscopy, teased preparation and electronic microscopy.

Materials and methods

Experimental animals

The experiment was performed on 40 Wistar albino rats, approximately 2 months old and weighing around 200 g. They were fed on standard instant food for laboratory rats, ad libitum, and watered from the city water supplies.

The animals were separated into two groups, the exposed and the control ones, 20 rats in each. Before the treatment, they went through a 14-day quarantine period.

The method of organic solvent application

The exposed animals inhaled the following mixture of organic solvents: toluene (C₆H₅CH₃) 30%, xylene C₆H₄(CH₃)₂ 30%, n-butyl-acetate (CH₃COCH₃) 10% and white spirit (also known as industrial petrol or petroleum) 10% (which is, by itself, a mixture of 80-86% saturated hydrocarbons and 1% olephine). Industrial solvents produced by paint and varnish factory "Pomoravlje", Niš, Yugoslavia, were used, while the ratio of the mixture components represents a model of working environment with multiple violations of concentration threshold levels. The total solvent quantity in the chamber air was 3,000
ppm. The animals were exposed 6 hours a day, 5 days, excluding weekends, during 8 weeks.

**Description of experimental apparatus**

Experimental chamber with additional devices and measuring instruments is depicted on Fig 1. The chamber (7) was designed as a glass cage equipped by the necessary switches for the air to flow in and out, as well as the measuring instruments for monitoring microclimatic conditions. The volume of the chamber is 1.5 m$^3$, while the flow of 15 m$^3$/h was maintained throughout the experiment, i.e. the air was changed 10 times an hour. Fresh air, previously filtered, was brought into the chamber by means of a compressor (1) with fluid separation, while the air flow was controlled by a rotometer (5). The air was induced into the aerosol-generator containing the mixture of organic solvents in the dose sufficient for the 6-hour period (1,310 ml). The air comes out of the aerosol-generator mixed with the dispersed solvent particles in the form of mist. Before entering the chamber, the mixture of air and solvents was induced into the cyclone separator in order to separate large drops of solvent mixture. The inflow of the mixture of air and solvents was introduced from the bottom side, by means of a distributor (6), which allows the flow of the prepared mixture throughout the whole chamber volume. The mixture outflow was made possible at the top of the chamber by means of a fan (8).

The subpressure of 120 Pa was maintained inside the experimental chamber in order to prevent the penetration of the air and mixture into the laboratory environment. The subpressure was reached by means of vacuuming fan with changeable rotation speed controlled by an open U-tube (12) with one end connected to the switch (14) in the chamber wall. The opposite chamber wall had a special switch (13) for obtaining air samples, in order to monitor and control the concentration level inside the chamber.

Monitoring of the parameters of the air brought into the chamber, as well as the air inside the chamber (microclimatic conditions - temperature, relative wetness, atmospheric pressure) is made possible by standard measuring instruments - thermometer (9), hygrometer (10) and barometer (11).

**Determination of mixture components by the "head-space" GC method in the air samples from the experimental chamber**

Air samples were taken from the experimental chamber: - in the "head-space" bottles, 10 ml each; - on the absorbent tubes with active coal and - in "supelco" gas cylinder, 20 ml each;

- The original mixture of organic solvents (acetone, toluene, n-butyl-acetate, xylene and white spirit in the ratio 10%:30%:20%:30%:10%) was also sampled, as well as individual organic solvent from the mixture contained in the tank of the aerosol-generator.

- Gas chromatograph with "head-space" gas injector; glass filled column 2 m × 0.4 mm based on absorbens Poparak -S; and fire-ionisation detector (FID); PERKIN-ELMER SIGMA 1B and integrator SIGMA 10B. Gas carrier-nitrogen; hydrogen from General-electric generator; air from the compressor of the institution.

The content of air samples (air from the sample vessels and from the active coal in the absorption tubes) was put into the "head-space" bottles with a hermetrical lid. After this the bottles were pre-heated for 5 minutes at 150°C, and subsequently exposed to nitrogen super-pressure from the gas injector for 1 minute. Immediately after this, the atmosphere from the bottle was injected into the gas-chromatograph analyzer. Gas chromatograph was recorded on the integrator plotter, together with the records of the surfaces of obtained chromatograph peaks of the particular monitored substances.

Result quantification was performed by the "external" method, according to the peak surfaces of the analytical standard from the original mixture, as well as according to the peaks of the analytes themselves in the examined samples. NB: the concentrations of white spirit could not be monitored at the above mentioned GC operational parameters. Operating conditions of the gas-chromatograph system: injector temperature -230°C, detector temperature 250°C, column temperature programme 160°C (7 minutes) up to 195°C (35 minutes), with temperature increasing at the pace of 10°C/min.

Detector sensitivity range = 1, surface sensitivity = 400, base sensitivity = 20, plotter-signal jam = 6 to 10. Paper 3 mm/min. Nitrogen pressure in the injector = 100 kPa, air pressure = 200 kPa, hydrogen pressure =
175 kPa, nitrogen flow through the column = 40 ml/min.

The identities of the examined substances from the gas mixture were determined by individual injection of certain analytical standards and by comparing retention times at the column of gas chromatograph.

**Method of victimization, sampling and processing the materials**

The rats were victimized in small groups, by decapitation.

**Pathohistological processing of the materials**

Approximately 3 cm long samples of peripheral n.ischiadicus were taken. Following a short-term fixation in 3.5% glutaraldehyde immediately after sampling, each sample was split into three parts. The first part, about 1 cm long, was cut longitudinally and again fixed in glutaraldehyde for several hours, then cut appropriately, additionally fixed, and having been washed in the phosphate buffer for several hours, it was post-fixed in 1% osmium tetroxide and then routinely cast in epon for semi-thin and ultra-thin samples in order to be examined by transmission electronic microscopy (TEM).

The second part, about 1 cm in length was prepared for teased preparation by appropriate fixation in glutaraldehyde and osmium tetroxide by usual laboratory routine.

After fixation in 10% formaline for at least 48 hours, the third part was routinely cast in paraffine in order to prepare longitudinal and cross-sectional samples, routinely stained by special methods for nerve tissue.

For epon embedding, 7 samples were used – 3 horizontally and 4 vertically oriented ones, for longitudinal and cross-sectional semi-thin (1 micron) sections. Semi-thin sections were stained by 0.1% of blue toluidine in boric acid and 1% solution of paraphenylenediamine. Ultra-thin sections were stained by uranyl acetate and lead citrate.

By teased preparations, individually or in small groups of several fibres, at least 100 fibres were obtained from each sample.

**Results**

**Histopathological results**

Longitudinal and cross-sectional samples of n.ischiadicus from paraffin casts do not show reliable pathological changes, except for the possible mildly to moderately pronounced endoneural edema, in some clusters. Importantly, no visible myelin changes were recorded by light microscopy.

Teased preparation of the most fibres, also does not show any visible pathological changes. A small percentage of fibres (around 5%) show oval or round focal myelin swellings up to 30 microns in diameter, with more intense staining than the rest of the myelin (Fig. 2). Considering fibres with at least 4 internodal segments, usually only one swelling of this type was seen, while fibres with 2 or 3 swellings are extremely rare. Very rarely, in some magnified fibres a barely visible, slightly lined myelin sheath can be perceived.

An inspection of semi-thin sections of epon blocks shows that the number, i.e., the density and the distribution of myelin fibres correspond to a normal n. ischiadicus of a rat (Fig. 3). The cross-sections show various suspect or positive pathological axonal or myelin sheath changes (Fig. 3 and 4). Most frequently, myelin sheath shows focal points in the shape of wrinkled, spirally arrayed myelin lamellae, facing the inner part or, very rarely, the outer part, various fissures, separation of myelin lamellae, as well as focal separations with reduplication. These changes are perceived in all clusters, but are not equally distributed – they are more frequent in some clusters than the others. Generally speaking, they can be seen in 5-6% of the fibres and are obviously more frequent in the exposed rats than in healthy, control rats. Focal wrinkles with nodular myelin formations exert compression upon the axons, so that, apart from minor or major deformation, they also show axonal degeneration in some fibres. A cross-section of one fibre displays 2 or 3 changes of this kind (Fig. 3 and 4).
Based on an assessment of the shape of cross-sections, this change can be localized in all parts of the internodal segment: nodally-perinodally, paranodally, internodally. Some degenerated fibres are cross-sectionally seen as severely deformed but, also, increased axoplasm density can be observed, which is strongly stained, but individual fibres with distinct contours and borders of myelin sheath and axon are very rare, as well as highly atrophic axons, which are barely seen in some degenerated fibres. Fibres with darker axoplasm stains and normal myelin sheath appearance are very rare (Fig. 4), as well as fibres with pale myelin sheath colour and its atrophic or occasionally swollen axons (Fig. 4, arrow).

Electronic microscopy results

Most of the fibres have a usual, normal appearance. However, fibres showing certain, possibly pathological changes or of disputable origin are not rare, with apparent changes in density and distribution of axoplasm organelles, fissures within myelin sheath or separation of myelin lamellae, occasionally clearly visible both in the regions of Schmidt-Lanterman incisions, and out of them (Fig. 5).

Frequently, this separation is accompanied by axonal changes, which cannot be interpreted as normal or disputable changes or artifacts, since these shapes are rarely or hardly ever seen in normal nerves. Clear degenerative myelin and axonal changes are of polymorphous nature. Both light microscopy, which proves nodular myelin changes compressing the axons, and electronic microscopy record tomaculum-like body reduplication of myelin lamellae, expressed multiply or as compressing highly atrophic axons within the whole circumference, showing increased density of organelles (neurotubules, neurofilaments, etc.) (Fig. 6).

Sometimes, these formations show several layers (Fig. 7). Superficial lines and tomaculous formations are rare, but sometimes 2 or 3 of them can be found on the fibre cross-section (Fig. 8). Axons of these fibres usually display no or mild changes. Non-myelin fibres do not show any visible or significant pathological changes, they have normal configuration in smaller groups (Fig. 7).
some Schwann cells of myelin fibres contains a highly proliferated endoplasmic reticulum channel network with numerous container-like outlets (Fig. 10). The cytoplasm of some Schwann cells shows signs of mitochondrial accumulation, mainly in the perinodal region (Fig. 11, arrows).

Fig. 8. EM. Three tomaculous formations on the surface of myelin sheath (arrows), whose axon (A) shows mild atrophy, ×12 000

Fig. 9. EM. Wide separation of the deformed and degenerated axon (A) from the myelin sheath (longer arrows). Mitochondria (shorter arrows) are accumulated in the cytoplasm of Schwann cells, ×7 000

Fig. 10. EM. Intensively developed endoplasmic reticulum and numerous vacuolae (V) in the cytoplasm of a myelin fibre Schwann cell, ×12 000

Fig. 11. EM. Accumulation of mitochondria in the cytoplasm of a myelin fibre Schwann cell paranodally, ×16 000

Discussion

Neurotoxic effects were examined on N.ischiadicus in rats exposed to an experimental mixture of organic solvents (toluene, xylene, n-butyl acetate, acetone and white spirit) in the concentration of 3,000 ppm, during 8 weeks, determining distal axonopathy-type changes.

During daily exposure, the rats showed signs of irritation and disturbed coordination followed by falling asleep after 2-3 hours, however, their recovery came quickly after the exposure cessation. After eight weeks, some loss of weight was noticed in experimental rats – 15%, compared to the control ones. All the experimental rats survived and were seemingly healthy, with possible, hardly noticeable ataxic movements (this remark has not been supported by any objective measurement results).

In comparison with numerous clinical studies, pathological-anatomical research is rarely found in the references. An overview of available data about the experiments of sub-chronic duration conducted on rats, in order to examine the effects of neurotoxicity induced by inhalation, suggests that the most frequent breeds of experimental rats were Wistar, Fischer, gerbils and Sprag-Dolley. The most frequent solvent concentrations in the air of experimental chambers ranged from 800 to 4,000 ppm. The range of daily exposure was four, six, eight, but not more than fourteen hours, over the total period of 30 days at the minimum, in order to develop toxic axonopathies (2,6,7,8,13). Experimental studies with hexa-carbonic mixtures, n-hexane, methyl-n-butyl ketone (MBK), 2,5-hexadiene (n-hexane major product), found in solvents most frequently used by car-varnishers, have proved their role in causing polineuropathies in both humans and animals (4,12). Experimental neuropathies caused by the above-mentioned solvents, as well as human "solvent"-type neuropathies are the evidence of the modifications of "dying-back" distal axonopathy. "Dying-back" degeneration model is the cause of clinically similar sensory-motor neuropathies. However, this group of disorders can include some nosological differences. Some distal axonopathies develop...
more slowly than others, some of them cause more impairment on sensory fibres, than motor ones, some of them are more likely to impair certain nerves (lead usually affects radial nerve, carbon-sulphide affects peroneal one, tri-chloro-ethylene impairs trigeminal nerve, sometimes central nervous system is affected rather than the peripheral one, which is why the term central-peripheral axonopathies was induced, as suggested by an American group of authors, notably Spencer and colleagues (2,4,12)). The initial changes occur in the branches of tibial nerve and, mainly, ischiadic stem, as a consequence of the effect of various chemical substances and some metabolic abnormalities (1,12). Giant focal swellings can be observed on axons, often 3-4 times larger in diameter than the largest axon, caused by an accumulation of neurofilaments; thus, the larger the axon, the thinner the myelin sheath becomes. As the paranodes become smaller and due to myelin retraction, the Ranvier nodes become bare, with the tendency of Schwann cells to migrate into the widened internodal space. In a number of experimentally-caused neuropathies, tomaculous thickening of myelin, as well as intraneural deposits of glycogen particles, can be observed.

With regard to these findings, the following experiment was also set: male Wistar rats (the exposed group) were constantly placed in the experimental chamber with the controlled micro-climatic parameters. A mixture of organic solvents, prepared in advance, was injected into the chamber daily, through a compressor and aerosol-generator for six hours, five days a week. The semi-thin cross-sections of epontic casts did not show any significant differences in the number, i.e. density, and the distribution of the diameters of the myelin nervous fibres. The cross-sections show visible possibly, but also some certainly pathological axonal and/or myelin sheath changes, which are significantly more frequent than in healthy, control rats. Focal wrinkling with a formation of nodular myelin thickening compresses the axons, causing them both to deform and degenerate. Fibre cross-section shows 2-3 tomaculous swellings, localized on all parts of the internodal segment: nodularly – perinodally, paranodally and internodularly. Fibres with a barely visible atrophic axon or those with darker axoplasm and normal appearance of the myelin sheath or pale myelin sheath and atrophic enlarged axons are rare.

A detailed ultrastructural analysis of toxic distal axonopathies can be done by electronic microscopy. Electromicroscopical examination of the ischiadic nerves of the rats intoxicated by the experimental mixture of organic solvents, predominantly, had normal. However, fibres which show certain pathological changes, such as the changes in the density and the distribution of myelin, fissures beneath the myelin sheath or a separation of myelin lamellae in and out of the region of Schmidt-Lanterman incisures are very commonly seen (Fig. 4). Frequently, this separation is accompanied by axonal changes (Fig. 5), so that it cannot be interpreted as normal or suspect change or artifact (due to preparatory procedure). Clear degenerative myelin and axonal changes are polymorphous. Tomaculous reduplication of myelin lamellae is sometimes expressed multiply or within whole circumference, compressing the highly atrophic axon which shows the increasing density of the organelles (neurofilaments, neurotubules etc.) or more severe changes with accumulating homogenous or grain-like material, as well as other structures of different electronically displayed density and shape (Fig. 6). Some axons show rough degenerative changes with axolemma swellings (arrows), with mild or no myelin sheath changes. Sometimes, these formations show different layers (Fig. 7). Lines and tomaculous formations on the outer surface of the myelin sheath are not so common, but occasionally two or three of them can be seen on a fibre cross-section (Fig. 8). The axons of these fibres usually show mild or no changes. Axonal separation from myelin sheath is frequent, especially in its initial forms (Fig. 4). Axons separated in this way, as well as fluid accumulation in these areas cause severe axonal deformities with their degeneration, sometimes accompanied by rough axoplasm vacuolization (Fig. 9, arrows). In addition to a well-pronounced Golgi apparatus, the cytoplasm of some Schwann cells of the myelin fibers sometimes contains a highly proliferated network of endoplasmic reticulum channel, as well as numerous cisternal outlets of the endoplasmic reticulum (Fig. 10). The cytoplasm of some Schwann cells also shows mitochondrial accumulation, predominantly in the par anodal region (Fig. 11, arrows). Non-myelin fibers do not suggest any visible pathological changes (Fig. 4).

In the course if their ultra structural and morphometric study of the biopsies of sural nerves of the workers exposed to organic solvents (minimum 15 years of working with varnishes) and healthy volunteers, Bertold et al., 1983, determined the presence of myelin fiber hypertrophy, a severe disorder of par anodal myelin sheath with tenacious myelin formations, as well as numerous mitochondria in the Schwann cells rich with "glycogen-like particles". This finding was also sporadically present in the control group varnishes, mostly older than 49. "Glycogen-like particles" are named liked this, because their poly-saccharide nature was not determined through enzyme digestion, although, ultrastructurally, they match monoparticular glycogen. Such particles were found in the spinal ganglions of pre-hibernating frogs, in the pre-clinical cases of metachromatic leukodystrophy and experimental diabetes, as well as in older rats (2). Berthold and colleagues interpret these "glucogen-like particles" as an early, non-specific sign of axonal pathology related to organic solvent exposure, i.e., they believe that such state enhances the effect of mechanical trauma (which is a typically accompanying noxa in these workers), and/or ageing. This large scale neuropsychiatric study of Berthold did not record any other neurophysiological deviations, apart from the ones mentioned above. Thus, a detailed structural analysis of peripheral nerves represents the most sensitive way of determining early impairments.

Lj. Blagojević, J. Blagojević, S. Dožić, S. Ćušić
Conclusion

An intoxication of male Wistar rats by inhalation of a mixture of organic solvents (toluene, xylene, n-butyl acetate, acetone and white spirit) in the concentration of 3,000 ppm, six hours a day, five days a week, during eight weeks resulted in the appearance of some initial changes, indicating toxic neuropathy, i.e., axonal degeneration accompanied by neurofilament accumulation of soft membrane profiles and mitochondria, which was confirmed by:

- light microscopic changes expressed as mildly to moderately pronounced endoneural edema, predominantly in the central parts of the rays and subperineurally, but not in all rays in the cross-sections and longitudinal sections. Light microscopy did not show any myelin changes.

- "teased" preparation which showed some focal oval or round myelin swellings, with axonal compression and occasional degeneration caused by the mentioned tomaculous swellings, as well as fibres with pale myelin sheath or swollen axons;

- electronic microscopy showed clear degenerative myelin changes in the form of tomaculous reduplication of myelin lamellae, sometimes multiply within the whole circumference compressing highly atrophic axons. The axons showed the increasing density of the organelles (neurofilaments and neurotubules, proliferated network of endoplasmic reticulum channel with numerous cysternal outlets), as well as mitochondrial accumulation in the cytoplasm of Schwann cells in the paranodal region.

References

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pojedinačnim vlknima "teased" preparaciji zapažena su fokalna ovalna ili okruglasta zadebljanja mijelina, sa kompresijom na aksone i mjestimičnom degeneracijom. Elektronskom mikroskopijom uočene su jasne degenerativne promene mijelina u vidu "tomaculum like body" redupliciranja mijelinskih lamela, ponegdje multiplno u celoj cirkumferenciji sa kompresijom na jako atrofične aksone, sa zgušnjavanjem organela (neurofilamenata i neurotubula, proliferisanog endoplazmatskog retikuluma) kao i akumulacijom mitochondrija u citoplazmi Schwann-ovih čelija u paranodularnoj regiji.

Ključne reči: N. išijadikus, aksonalna degeneracija, organski rastvarači, pacovi