Values of markers of early and advanced glycation and lipoxidation in serum proteins of children with diabetes mellitus

Jakus V, Bauerova K, Michalkova D, Carsky J

Hodnoty markerov skorej a pokročilej glykácie a lipooxidácie sérových proteínov u detí s diabetes mellitus

Abstract

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Background: **Advanced glycation endproducts (AGEs) have been established as one of the major factors responsible for the multi-organ damage seen in diabetes. AGEs and lipoxidation products, as e.g. MDA, and their adducts with proteins appear to be formed together in serum and tissues. A link between AGEs formation and increased lipoxidation at tissue damage is under investigation.**
Aim: **The aim of the present study was to determine fructosamine (FAM), glycated haemoglobin (HbA_1c) AGEs-specific fluorescence and MDA-protein adducts specific fluorescence in diabetic and in healthy children, with statistical evaluation of the relationship between the parameters assessed.**
Subjects and methods: **Values of FAM and HbA_1c (spectrophotometry) and of AGEs-specific fluorescence and MDA-protein adducts specific fluorescence were investigated in serum proteins of 17 children with poorly controlled type 1 diabetes mellitus (age range 9 to 18 years). Eight healthy children (age range 7 to 17 years) served as controls.**
Results: **In the diabetic group, all the parameters evaluated were significantly higher than in the control group. Furthermore, MDA-linked specific fluorescence of MDA-protein adducts (a biomarker of oxidative stress) was correlated with AGEs-specific fluorescence. In patients this correlation was extremely significant (r=0.8176, p<0.0001).**

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Abstrakt

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Hodnoty markerov skorej a pokročilej glykácie a lipooxidácie sérových proteínov u detí s diabetes mellitus
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Pozadie problému: Koncové produkty pokročilej glykácie (AGE produkty) sa ukázali ako jeden z hlavných faktorov zodpovedných za multiorgánové poškodenie pozorované pri diabete. AGE produkty a produkty lipooxidácie, ako napríklad malóndialdehyd (MDA) a ich adukty s proteínmi sa spolu tvoria v sére i tkanivách. V súčasnosti sa intenzívne študuje prepojenie medzi tvorbou AGE produktov a zvýšenou lipooxidáciou pri poškodení tkaniva.
Cieľ: Určiť fruktozamín (FAM), glykovaný hemoglobín (HbA_1c), špecifickú fluorescenciu AGE produktov a MDA-proteínových aduktov u diabetických a zdravých detí, so štatistickým vyhodnotením vzťahu medzi nameranými parametrami.
Subjekty a metódy: Hodnoty FAM, HbA_1c, špecifickej fluorescencie AGE produktov a MDA-proteínových aduktov sa vyšetrovali v sére u 17 detí so zle kontrolovaným diabetom typu 1 (vek 9—18 rokov). Osem zdravých detí (vek 7—17 rokov) bolo vybraných ako kontrola.
Výsledky: Všetky skúmané parametre boli signifikantne vyššie pri diabetickej skupine oproti kontrole. Špecifická fluorescencia MDA-proteínových aduktov (biomarker oxidačného stresu) korelovala so špecifickou fluorescenciou AGE produktov. U diabetických detí táto korelacia bola veľmi významná (r=0,8176, p<0,0001).

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Conclusion: **The increased oxidative stress in children with type 1 diabetes may not be attributed to complications, though it could contribute to the development of complications.** ***(Tab. 2, Fig. 7, Ref. 30.)***

Key words: advanced glycation, AGEs, lipoxidation, MDA-protein adducts, diabetes mellitus, children.

Záver: Zvýšený oxidačný stres u detí s diabetom typu 1 nemožno vzťahovať ku komplikáciám, mohol by však prispievať k vývoju neskorších komplikácií. *(Tab. 2, obr. 7, lit. 30.)*

Kľúčové slová: pokročilá glykácia, AGEs, lipooxidácia, MDA-proteínové adukty, diabetes mellitus, deti.

The high incidence of vascular complications in patients with diabetes mellitus is poorly understood. Results of the Diabetes Control and Complications Trial (1993) established that prolonged exposure to hyperglycaemia is to be considered the primary factor associated with the development of diabetic microvascular complications in type 1 diabetic patients. The Diabetes Control and Complications Trial (1993) and the UK Prospective Diabetes Study (1998) showed that improved glycaemic control, as measured by reduction of glycated haemoglobin (HbA_1c), significantly reduced the risk of development and/or progression of all diabetic complications.

Several metabolic or endocrine abnormalities have been postulated as possible triggers for micro- and macroangiopathies. A number of hypotheses regarding the link between hyperglycaemia and diabetic complications have equally strong support among diabetes researchers and clinicians (Feener and King, 1997). Among these are hypotheses based on the deleterious effects of altered aldose reductase (polyol pathway), protein kinase C, growth factor, cytokine activities, oxidative, including carbonyl stress (Baynes, 1991; Baynes and Thorpe, 1999).

The glycation or Maillard (chemical) hypothesis proposes that complications in diabetes are a direct consequence of accelerated, cumulative modification of proteins and other biomolecules by glucose or its metabolic intermediates during hyperglycaemia in diabetes. The glycation process in vivo results in two different products: early and advanced glycation endproducts (AGEs). The mechanism of early glycation (Amadori) product formation has been well described, with glycated haemoglobin (HbA_1c) as the best studied example (Rahbar, 1980; Rácz et al., 1989). HbA_1c has been well established as an important indicator for glycaemia monitoring. Another example of Amadori product is Amadori albumin. Experimental and clinical studies showed that Amadori albumin was associated with nephropathy and exerted a pathophysiological role in microvascular diabetic complications (Schalkwijk et al., 1999). AGEs may arise by several mechanisms (Scheme 1) (Vlassara, 1997; Jakuš and Rietbrock, 1999). AGEs have been established as one of the major factors responsible for the multi-organ damage seen in diabetes and aging (Brownlee, 2000; Jakuš, 2000).

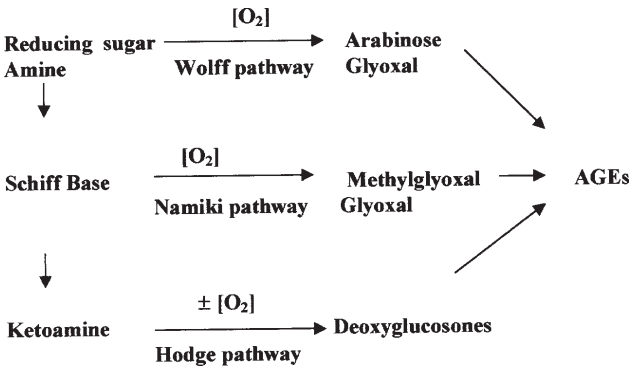
AGEs and lipid peroxidation products (MDA, 4-HNE, acroleine) and their adducts with proteins appear to be formed together in serum and tissues (Miyata et al., 1998). The report of Sajithlal and Chandrakasan (1999) provides a new possible link between increased lipid peroxidation and tissue damage through AGEs. Interaction between lipoxidation products and the long-lived structural protein, collagen, results in the formation of products (crosslinks) that are structurally and immunologically similar to AGEs. These results suggest that, in addition to reducing sugars, fatty acid oxidation products can also lead to the formation of products that are structurally similar to AGEs.

Another study demonstrated that one AGE product — carboxymethyllysine — could be derived not only from carbohydrates, but also from oxidation of polyunsaturated fatty acids in vitro (Miyata et al., 1998). In an experimental model, Traverso et al. (1998) showed immunological evidence for increased oxidative stress in diabetic rats, supporting the hypothesis of increased oxidative damage in diabetes. The levels of immunogenic MDA — rat serum albumin adducts measured by ELISA — highly correlated with relative fluorescence at 390 nm excitation/460 nm emission, considered specific for MDA — protein adducts. Several studies (Dominguez et al., 1998; Diabetes Control and Complications Trial, 1993; UK Prospective Diabetes Study, 1998; Jain et al., 1989) showed that in diabetes increased blood concentrations of markers of oxidative stress, especially in patients with poor glycaemic control, have been implicated in the development of vascular complications.

The aim of the present study was to determine fructosamine (FAM), HbA1c, AGEs-specific fluorescence and MDA-protein adducts specific fluorescence in 17 diabetic and 8 healthy children, with statistical evaluation of the relationship between the parameters assessed.

Subjects and methods

The subjects in this study were selected from a group of patients regularly attending the Department of Paediatrics, University Hospital, Comenius University. To be eligible to enter the stu-



Scheme 1. Pathways of AGEs formation. Wolff pathway — glucose may undergo metal-catalysed autoxidation to produce reactive carbonyl precursors of AGEs, Namiki pathway — Schiff bases formed on reaction of glucose with protein, undergo reverse aldol reactions and autoxidative cleavage to produce AGE precursors, Hodge pathway — AGE precursors are formed by rearrangement and autoxidation of the Amadori product. Schéma 1. Cesty tvorby AGE produktov. Wolffova cesta — glukóza môže podliehať kovom katalyzovanej autooxidácii za tvorby reaktívnych karbonylových prekurzorov AGE produktov, Namikiho cesta — Schiffove bázy tvorené reakciou glukózy s proteínom podliehajú reverzným aldolovým reakciám a autooxidačnému štiepeniu za tvorby AGE produktov, Hodgeho cesta — AGE prekurzory sa tvoria prešmykom a autooxidáciou Amadoriho produktu.

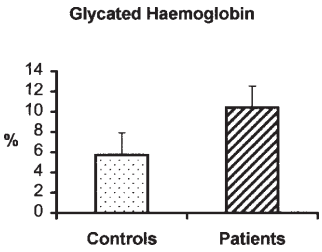


Fig. 1. Comparison of HbA_1c in diabetic patients vs controls: extremely significant difference, p<0.0001. Results are presented as mean and SD.
Obr. 1. Porovnanie HbA_1c u diabetických pacientov vs kontrola: veľmi významný rozdiel, p<0,0001. Výsledky sú prezentované ako priemer a smerodajná odchýlka.

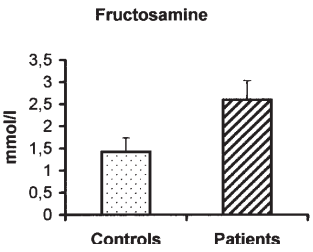


Fig. 2. Comparison of FAM in diabetic patients vs controls: extremely significant difference, p<0.0001. Results are presented as mean and SD.
Obr. 2. Porovnanie FAM u diabetických pacientov vs kontrola: veľmi významný rozdiel, p<0,0001. Výsledky sú prezentované ako priemer a smerodajná odchýlka.

dy, patients had to be 9 to 18 years of age, have type 1 (insulin-dependent) diabetes as defined by the National Diabetes Data Group (1979) and no clinical signs of microvascular complications (retinopathy, nephropathy, neuropathy). Seventeen children with diabetes satisfied these inclusion criteria: 6 were <12 years old (group 1, prepubertal patients) and 11 were >12 years old (group 2, pubertal patients). All seventeen patients had poor glycaemic control (defined as $HbA_1c \geq 9$ %). Eight age-matched healthy subjects served as controls. The number of subjects included in this study, with pre-school children excluded, was limited to respect the requirements of the local ethical committee.

Blood, serum and urine were obtained from children with diabetes and from control subjects. The biological samples were stored at -20 °C to reserve their adequate stability.

Measurements of early glycation products

The values of FAM were determined photometrically by Roche Kit (Johnson et al., 1983). The values of HbA_1c were determined photometrically at 415 nm by using the kit Sigma Diagnostics No. 442-B (Rahbar, 1980).

Measurements of s-AGEs by AGEs-linked specific fluorescence

Serum was diluted 20-fold with phosphate buffered saline. The fluorescence intensity of serum was measured at 418 nm after excitation at 346 nm (Yanagisawa et al., 1988), using a fluorescence spectrophotometer Kontron SFM 25. Fluorescence was expressed as the relative fluorescence intensity in arbitrary units (A.U.). Chinine sulphate was used to calibrate the instrument and monitor its performance.

Measurement of MDA as MDA-protein adducts-linked specific fluorescence

Serum was diluted 20-fold with phosphate buffered saline. The fluorescence intensity of serum was measured at 460 nm after excitation at 390 nm (Odetti et al., 1994), using a fluorescence spectrophotometer Kontron SFM 25. Since a MDA-protein adduct (Odetti et al., 1994) as a synthesised standard was not available, MDA-protein adducts specific fluorescence was expressed as the relative fluorescence intensity in p.c., where a healthy donor represented 0 % fluorescence and a diabetic child with the highest AGEs-specific fluorescence represented 100 % fluorescence.

Statistical calculations were performed using GraphPad Instat tm program. Unpaired Student's t-test (two-tailed) and Pearson linear correlation were used. For a p value less than 0.0001 the statistical significance was defined as extremely significant.

Tab. 1. Individual values of FAM, HbA_1c, s-AGEs and MDA-protein adducts in healthy children.
Tab. 1. Individuálne hodnoty FAM, HbA_1c, s-AGEs and MDA-proteínových aduktov u zdravých detí.

Subject	FAM (mmol/l)	HbA_1c (%)	s-AGES (A.U.)	MDA-protein adducts (%)
1	1.38	6.18	17.2	27.0
2	1.06	5.90	21.6	0 (min)
3	1.18	4.40	16.4	22.9
4	1.08	4.25	17.2	41.4
5	1.63	4.50	17.8	28.0
6	1.33	4.85	20.8	28.5
7	1.92	10.9	15.1	28.2
8	1.70	12.75	17.7	17.1

Results

The individual values of all the evaluated parameters are given in table 1 for healthy children and in table 2 for children with diabetes. For the latter group also the values of glycaemia are recorded.

In the diabetic group, HbA_1c (Fig.1), FAM (Fig.2), AGEs (Fig. 3) and MDA-protein adducts (Fig.4) were higher than in the control group. The calculated p values were all less than 0.0001, therefore the means of the assessed parameters differ extremely significantly between the diabetic and control group. Furthermore, MDA-linked specific fluorescence of MDA-protein adducts (a measure of oxidative stress) was correlated with AGEs-specific fluorescence. In patients (Fig. 5) this correlation was extremely significant ($r=0.8176$, $p<0.0001$). In the control group (Fig. 6) MDA failed to correlate with s-AGEs ($r=-0.5475$, $p=0.1601$).

Discussion

The FAM assay is commonly used for measuring glycation of plasma proteins and provides a short-term (7—10 day) index of

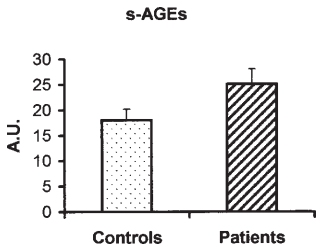


Fig. 3. Comparison of AGEs in diabetic patients vs controls: extremely significant difference, p<0.0001. Results are presented as mean and SD.
Obr. 3. Porovnanie AGEs u diabetických pacientov vs kontrola: veľmi významný rozdiel, p<0,0001. Výsledky sú prezentované ako priemer a smerodajná odchýlka.

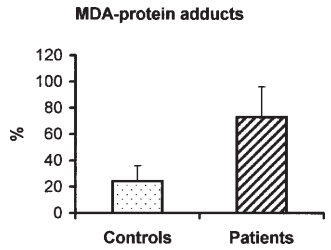


Fig. 4. Comparison of MDA-protein adducts in diabetic patients vs controls: extremely significant difference, p<0.0001. Results are presented as mean and SD.
Obr. 4. Porovnanie MDA-proteínových aduktov u diabetických pacientov vs kontrola: veľmi významný rozdiel, p<0,0001. Výsledky sú prezentované ako priemer a smerodajná odchýlka.

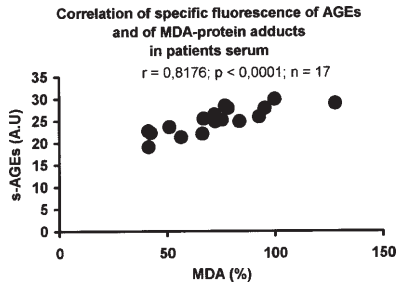


Fig. 5. Scatterplot showing the correlation between s-AGEs specific fluorescence and MDA-protein adducts specific fluorescence in serum of patients.
Obr. 5. Scatterov diagram zobrazujúci koreláciu medzi špecifickou fluorescenciou pre AGE produkty a špecifickou fluorescenciou pre MDA-proteínové adukty v sére pacientov.

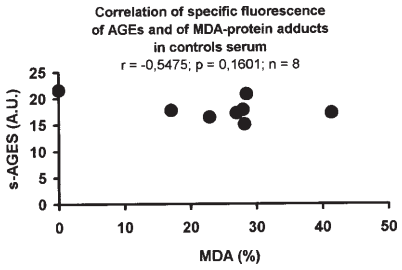


Fig. 6. Scatterplot showing the correlation between s-AGEs specific fluorescence and MDA-protein adducts specific fluorescence in serum of healthy children.
Obr. 6. Scatterov diagram zobrazujúci koreláciu medzi špecifickou fluorescenciou pre AGE produkty a špecifickou fluorescenciou pre MDA-proteínové adukty v sére zdravých detí.

glycaemic control. A kinetic FAM assay has been adapted to automated analysis, making it a convenient tool for assessing recent glycaemic control (Johnson et al., 1983).

Measurements of HbA_1c are used routinely for clinical management of diabetes (Hudson et al., 1999). Haemoglobin, like the red blood cell, has a longer half-life than plasma proteins, i.e. a 3—4 month lifespan in the circulation. Since glycation is a slow reaction, the extent of glycation of haemoglobin depends on long-term mean blood glucose concentration. In practice, the percentage of haemoglobin glycation correlates most strongly with the mean blood glucose concentration assessed during the preceding 4—6 weeks. Normally, about 4—7 % of haemoglobin molecules are glycated, depending on the assay used. This value may increase to 16 % or higher in metabolically poorly controlled diabetic patients. In light of the results of diabetes complication screening in children and adolescents (Donaghue et al., 1999), higher HbA_1c has also become increasingly recognised as a significant risk factor for the development of tissue complications. In our study both parameters, FAM and HbA_1c, were extremely significantly higher in comparison to controls (Fig. 1 and 2). This fact could speak for the high risk of developing diabetic complications.

Because of low AGEs in tissue proteins, specific and accurate measurements of AGEs require gradient HPLC analysis or gas or liquid chromatography-mass spectrometry (GC/MS). These assays are not readily adapted for routine use in the clinical laboratory. ELISA assays are now available, however, they are not widely used in clinical settings. According to Yanagisawa et al. (1998), we evaluated the circulated AGEs by measurement of AGE-specific fluorescence. The author recommended this method as a simple and useful test to assess AGEs both in blood and in urine. AGEs-specific fluorescence measurements in our patients showed that, compared with control subjects, children with type 1 (insulin-dependent) diabetes (duration of diabetes 12—121 months), free from microvascular complications, had markedly increased levels of s-AGEs (tables 1 and 2, Fig. 3). Thus s-AGEs might prove to be an early marker of potential late diabetic complications.

Many studies reported considerably increased MDA concentrations in patients with diabetes mellitus (Slatter et al., 2000). MDA modification of basic amino acid side-chains resulted in a change in its properties (Nair et al., 1981; Slatter et al., 1998; Traverso et al., 1998). Cross-sectional studies in young diabetic patients showed that systemic oxidative stress was present at the early onset of type 1 diabetes and increased by early adulthood.

Tab. 2. Individual values of glycaemia, FAM, HbA_1c, s-AGEs and MDA-protein adducts in patients.
Tab. 2. Individuálne hodnoty glykémie, FAM, HbA_1c, s-AGEs and MDA-proteínových aduktov u pacientov.

Subject	Glyc. (mmol/l)	FAM (mmol/l)	HbA_1c (%)	s-AGES (A.U.)	MDA-protein adducts (%)
1	7.50	2.79	9.66	25.5	67.0
2	17.0	2.67	9.95	22.7	41.4
3	20.8	2.04	11.5	23.6	51.2
4	22.0	2.09	12.5	22.3	42.5
5	19.3	2.33	11.9	24.9	83.6
6	13.0	2.79	10.55	27.9	95.4
7	24.0	3.11	13.25	25.9	92.7
8	7.50	2.45	12.35	22.1	66.5
9	9.20	2.35	8.70	19.1	41.6
10	10.4	2.96	10.35	29.9	100
11	14.9	3.08	13.0	26.4	72.0
12	10.0	2.96	11.9	25.2	75.6
13	17.5	3.00	8.95	21.3	56.6
14	15.9	2.70	9.20	24.9	72.4
15	9.50	2.50	10.1	27.9	78.2
16	21.2	2.87	8.65	28.9	128
17	4.80	1.48	11.6	28.4	77.0

Indicative parameters of lipid peroxidation and protein oxidation (MDA and protein carbonyl group levels) in plasma were progressively higher in diabetic children and adolescents than in control groups (Dominguez et al., 1998). Similarly in our study, enhanced values of MDA-protein adducts were found in children with poorly metabolically controlled diabetes (Fig. 4). The enhanced values of MDA-protein adducts significantly correlated with the enhanced AGEs (Fig. 5). It is important to monitor both glycoxidation and lipoxidation products, as suggested also by other authors in studies with serum albumin, red blood cell membranes, collagen, glomerular lesions (Nakayama et al., 1999; Lyons et al., 1997 a, b; Odetti et al., 1996; Suzuki et al., 1999) in poorly controlled diabetes. It appears plausible to recommend accurate measurements of oxidative stress, which can play an important role as an outcome measure in clinical studies in combination with data on AGEs levels.*

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PREDSTAVUJEME NOVÉ KNIHY

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Na knižný trh sa v letných mesiacoch roku 2000 dostala oddborná publikácia *Otravy hubami*, ktorej autorom je doc. MUDr. Peter Gavorník, PhD., renomovaný slovenský lekár internista-angiológ, ale aj odborník v oblasti lekárskej mykológie (mykotoxikológie).

Knihu *Otravy hubami* vydala Univerzita Komenského vo Vydavateľstve UK v rozsahu 100 strán. Monografia sa člení v podstate okrem predslovu a úvodu na štyri hlavné časti.

V predslove autor opisuje niektoré charakteristiky ríše húb a definuje jedlé, nejedlé a jedovaté huby.

V úvode opisuje originálnu etiopatogenetickú klasifikáciu otráv hubami, ktoré rozdeľuje na tri hlavné skupiny: pravé otravy hubami (intoxicatio fungina vera), nepravé otravy hubami (intoxicatio fungina spuria) a pseudootravy hubami (pseudointoxicatio fungina).

V prvej časti venovanej pravým otravám hubami sa tieto otravy rozdeľujú na viacero podskupín: primárne a sekundárne. Primárne pravé otravy sa ďalej rozdeľujú na podskupinu, ktorá je vyvolaná termolabilnými toxínmi (otravy z nedodržania technológie kuchynskej tepelnej prípravy húb), a podskupinu, ktorá je vyvolaná termolabilnými toxínmi, pričom sa rozlišuje osem typov týchto otráv: 1. typ — cyklopeptidový, cytotoxický, II. typ — gyromitrínový, mono,etylhydrazínový, III. typ — muskarínový, IV. typ — koprínový, V. typ — izoxazolový, neurotoxický, VI. typ — indolový psychotropno-neurotoxický, VII. typ — gastrointestinálny, VIII. typ — orelanínový, nefrotoxický. Sekundárne pravé otravy hubami môžu vzniknúť po konzumácii jedlých húb pri autolytických procesoch v hubách alebo pri kontaminácii niektorými mikroorganizmami.

V druhej časti venovanej nepravým otravám hubami sa tieto otravy rozdeľujú na dve podskupiny. V etiopatogenéze primárnych nepravých otráv hubami sa uplatňuje najmä intolerancia a imunitné reakcie. V etiopatogenéze sekundárnych nepravých otráv hubami sa uplatňuje najmä poškodenie organizmu kovmi a ich zlúčeninami, ionizujúcim žiarením (rádionuklidmi), pesticídmi, kontaminácia mikroorganizmami a karcinogénny a mutagénny účinok.

V tretej časti autor upozorňuje aj na zdanlivé otravy hubami, pseudootravy hubami, čo sú chorobné stavy, ktoré vzniknú v časovej súvislosti, nie však v príčinnej súvislosti s požitím húb.

V štvrtej časti autor podrobne opisuje zásady primárnej, sekundárnej a terciárnej prevencie otráv hubami.

Text monografie je prehľadne členený, doplnený 10 tabuľkami a 16 obrázkami, ktorých zoznamy sú aj zvlášť uvedené, čím sa zrozumiteľnosť zvyšuje.

Kniha *Otravy hubami* predstavuje vedecko-odborné aj praktické, dlho chýbajúce dielo, užitočné predovšetkým v pregraduálnej a postgraduálnej výučbe študentov medicíny a lekárov, v akútnej medicíne, ale zároveň pútavé a zrozumiteľné dielo pre všetkých milovníkov prírody. Publikácia je veľmi prehľadne štruktúrovaná, na výbornej jazykovej úrovni v časti čisto mykologickej aj internisticko-toxikologickej, čo ocenia hlavne lekári pri riešení akútnych situácií v klinickej praxi. Osobitne treba vyzdvihnúť originálnu etiopatogenetickú klasifikáciu otráv hubami, ktorá je diferenciálnodiagnostickým algoritmom správnej a rýchlej diagnostiky a adekvátnej liečby. Slovenské a latinské zoznamy jedovatých húb umožňujú jednoducho zistiť, aký druh a typ otravy spôsobuje konkrétna huba. Docent Gavorník úspešne zúročil svoju dlhoročnú mykotoxikologickú konzultačnú činnosť a potvrdil, že je mimoriadne všestranným klinickým lekárom a vynikajúcim vysokoškolským pedagógom.

Kniha *Otravy hubami* je unikátnym dielom v oblasti našej lekárskej mykologickej a internisticko-toxikologickej literatúry. Svojou rozsiahlosťou a koncíznosťou nemá obdobu ani v dostupnej zahraničkej literatúre.

I. Bátora