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STEVIOL GLYCOSIDES, *L*-ARGININE, AND CHROMIUM(III) AFFECT TRACE ELEMENT STATUS IN DIABETIC RATS

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ABSTRACT

Background. Iron, zinc, copper, and chromium status is often disturbed in the course of metabolic diseases, including type 2 diabetes mellitus. This brings about alterations and abnormalities in the absorption, distribution, and excretion of certain elements and in turn, is responsible for further progression of the disease. This study aimed to evaluate the effects of a high-fat diet supplemented with a combination of steviol glycosides (stevioside or rebaudioside A), *L*-arginine (two levels), and chromium(III) (two levels) on trace element (Fe, Zn, Cu, Cr) content in the liver and kidneys of rats with induced type 2 diabetes.

Materials and methods. The experiment was carried out on 110 rats, of which 100 were induced with mild type 2 diabetes with high-fat diet feeding and intraperitoneal streptozotocin injection. Afterward, the diabetic animals were divided into 10 groups and received either a high-fat diet, a high-fat diet with metformin (0.3%), or a high-fat diet supplemented with a combination of steviol glycosides (stevioside or rebaudioside A, 2.5%), *L*-arginine (2% or 4%), and chromium(III) (0.001% or 0.005%) for 6 weeks. The Fe, Zn, Cu, and Cr content in tissues was determined after microwave mineralization of samples and then using the atomic absorption spectroscopy (AAS) method.

Results. Induced hyperglycemia disturbed several tissular trace element levels in the liver and kidneys of type 2 diabetic rats. Combined supplementary factors, such as the type of steviol glycoside or levels of either *L*-arginine and/or chromium(III), were able to mitigate some alterations of trace elements, while some particular combinations of experimental factors even increased certain trace elements content in the analyzed internal organs of rats.

Conclusions. Mild hyperglycemia disturbs trace element (Zn, Cr) balance by shifting trace element concentrations in the critical organs (liver, kidneys) in type 2 diabetes rats. Supplementary agents can independently, or in certain combinations, mitigate some trace element alterations or even cause further changes in their concentrations in the liver or the kidneys. The metabolic significance of these alterations is not fully understood and warrants further studies.

Keywords: stevia, steviol glycosides, *L*-arginine, chromium, trace elements, diabetes

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic, complex, and increasingly common metabolic disorder. It involves a combination of symptoms such as hyperglycemia, insulin resistance, and impaired insulin release. The burden of T2DM is attributed to a sedentary lifestyle, poor diet habits, and obesity (Ogurtsova et al., 2017). The dangerous nature of the condition is associated with an increased risk of cardiovascular

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diseases, neuropathy, kidney malfunctions and/or retinopathy. Most crucially, it significantly contributes to higher mortality in populations (American Diabetes Association, 2018). Management of T2DM depends on the overall patient health status, but commonly includes diet and physical activity modifications and pharmacotherapy (Davies et al., 2018). Nevertheless, available management strategies are insufficient because a multitude of individuals fail to achieve optimal glycemic targets (Bailey et al., 2016; Inzucchi et al., 2015). T2DM represents a complex and multifactorial public health challenge with profound implications for global health, thus one of the major research efforts nowadays is to develop a treatment that combines effectiveness, cost-effectiveness and low risk of side effects (Zhuo et al., 2014). Not only does it require primary prevention, early detection, and patient education, but, inter alia, a wide elucidation of the mechanisms of insulin resistance and/or beta-cell dysfunction and individualized modalities such as incretin-based therapies, sodium-glucose co-transporter 2 inhibitors, glucagon-like peptide-1 receptor agonists, or finding novel sources of anti-diabetic agents (Davies et al., 2018; Drucker, 2016; Kurek and Krejpcio, 2019; Nauck, 2014).

A source of new substances whose health-promoting, including anti-diabetic, effects are currently under study is *Stevia rebaudiana* Bertoni, a small plant native to regions of present-day Brazil and Paraguay (Abudula et al., 2004; Jeppesen et al., 2003; Kurek et al., 2020; Kurek and Krejpcio, 2019; Lee et al., 2001; Mejia and Pearlman, 2019; Ray et al., 2020; Saravanan and Ramachandran, 2013; Wang et al., 2014). Steviol glycosides (SG) (most importantly stevioside (ST) and rebaudioside A (RA)) isolated from the plant are attractive and safe sweeteners commonly used in the food industry as they are intensely sweet, providing near-to-zero calories (Geuns, 2003; Koyama et al., 2003). The human body does not metabolize steviol glycosides (Gardana et al., 2003). In animals and humans, SG pass through gastrointestinal track intact and are enzymatically broken down into steviol and glucose by bacteria living in the colon. Released steviol is absorbed and transferred to the liver, where it is coupled with glucuronic acid to glucuronides, which are further excreted with urine through the kidneys. Results of experimental studies have shown that replacing sugar with stevia-based sweeteners may significantly reduce the overall calorie intake and even improve glycemic parameters (Anton et al., 2010; Gregersen et al., 2004).

L-arginine (*L*-arg) is a conditionally essential amino acid for humans. It is involved in many metabolic processes responsible for insulin sensitivity and vascular function regulation. As *L*-arg has its role in nitric oxide (NO) production and is involved in insulin signaling pathways (Jobgen et al., 2006), researchers have been investigating its potential in T2DM management over the last few years. It has been found that reduced NO bioavailability can contribute to impaired vascular health (Sansbury and Hill, 2014). *L*-arg is a substrate in the biosynthesis of NO by endothelial nitric oxide synthase. Therefore, it is a potentially beneficial agent in endothelial function improvement (Naseem, 2005). What is more, *L*-arg may also reduce blood pressure and improve insulin sensitivity, which is an additional advantage in the course of T2DM (Lucotti et al., 2006; Piatti et al., 2001). A few studies showed improvements in glucose metabolism in T2DM after *L*-arg supplementation, however, the results of most experiments are not clear (Jobgen et al., 2006). Responses to *L*-arg seem to be individualized and vary, thus it is difficult to determine the optimal dose and time of therapy, especially when additional doses of *L*-arg may be sometimes disadvantageous in some individuals (Bode-Böger et al., 1996; Szlas et al., 2022).

Chromium(III) (Cr(III)) is a trace mineral that plays an essential role in both glucose metabolism and the insulin signaling pathway. Research on this agent in individuals with T2DM has lasted for many years and explored the potential of Cr(III) supplementation (Vincent, 2000). Results of experimental studies suggest that chromium helps potentiate insulin signaling pathways and thus improves glucose uptake (Anderson, 1998). A potential link between chromium and insulin resistance has been found, since chromium deficiency was observed in patients with T2DM (Vincent, 2000). Chromium supplementation can reduce blood glucose levels and improves insulin sensitivity (Bahijiri et al., 2000; Cefalu and Hu, 2004). However, some trials showed minimal to no significant effects of this element on glucose management (Albarracin et al., 2008; Balk et al., 2007). Therefore, there is no definite optimal dosage of Cr(III) supplementation for

T2DM patients. Moreover, this element should be used with special caution as it can induce gastrointestinal discomfort in some patients and/or interact with other components of pharmacotherapy (Balk et al., 2007).

Body trace element status depends on a variety of external and internal factors. For example, it can be affected by genetic factors, dietary behaviors, and multiple health conditions. Iron, copper, and zinc status is often disturbed in the course of metabolic diseases, including T2DM, that bring about alterations and abnormalities in the absorption, distribution, and excretion of certain elements, which in turn is responsible for further progression of T2DM (Barbagallo and Dominguez, 2015). For instance, iron is essential for insulin pathway and it substantially affects gluconeogenesis (Harrison et al., 2023); copper chelators can improve vascular system function, reduce cell toxicity, regulate cuproptosis, and thus alleviate diabetic complications, such as cardiomyopathy, retinopathy, or neuropathy (Chang and Li, 2023); and suboptimal zinc intake and/or decreased zinc levels may correlate with insulin resistance and beta-cell dysfunctions in T2DM patients (Basaki et al., 2012; Jayawardena et al., 2012).

It has been hypothesized that both induced mild hyperglycemia and supplementary combined factors, such as the type of SG (ST or RA), *L*-arg, and Cr(III) levels can affect trace element levels in the critical organs (liver, kidney) in experimental rats. Thus, this study aimed to evaluate the effect of a high-fat (HF) diet supplemented with SG (ST or RA), *L*-arg, and Cr(III) on trace element (Fe, Zn, Cu, Cr) status in type 2 diabetic rats.

MATERIAL AND METHODS

Test reagents

Steviol glycosides (ST (≥98%, HPLC) and RA (≥98%, HPLC)) were purchased from Anhui Minmetals Development Imp. & Exp. Co. Ltd., Hefei, China. *L*-arg (99%, FCC) and STZ (≥98%, HPLC) were purchased from Sigma-Aldrich Sp. z o.o., Poznań, Poland. Cr(III) was used in the form of chromium(III) propionate complex (Cr3) [Cr3O(O2CCH2CH3)6(H2O)3]+(NO3) with the elemental chromium content of 19.5%, which was confirmed and supplied by the Department of Chemistry and Biochemistry of the University of Alabama (Tuscaloosa, AL, USA). Metformin hydrochloride was obtained from Metformax (Teva Operations Poland Sp. z o.o., Kraków, Poland), a commercially available prescription drug.

Course of the experiment

Type 2 diabetes was induced in the rats using a modified method presented by (Zhang et al., 2008).

The animals (110 Wistar rats, *Rattus norvegicus*) were purchased from Animalab Sp. z o.o. (Poznań, Poland). The access to food and drinking water was unlimited throughout the entire experiment. The experiment was conducted under controlled conditions in the animal facility of Poznań University of Life Sciences. The protocol was approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

At the outset, the animals were classified into two groups: healthy control (n = 10) (C) and T2DM group (n = 100) (Db). The standard AIN-93M diet was administered to the C group throughout the entire experiment. HF diet (40% of the caloric value derived from fat) was administered to the Db group for 7 weeks in order to induce insulin resistance. Then, multiple intraperitoneal STZ injections (20 mg/kg BW, 2–3 sessions with 3-day intervals) were performed to induce hyperglycemia in the Db group. Fasting blood glucose measures of at least 160 mg/dL in Db group animals were considered indicative of type 2 diabetes. Animals in the C group were given a placebo instead (pH 4.4 citrate buffer). The Db group was subsequently split into 10 different groups (by average fasting glucose level and body weight in each group), giving 11 experimental groups (n = 10) in total, along with the C group. Thereafter, the animals were administered diets enriched with specific combinations of supplements (ST/RA, *L*-arg, and Cr3) for 6 weeks, according to the following pattern:

- C group – standard AIN-93M diet throughout the entire experiment
- Db group – HF diet throughout the entire experiment
- Db + Met group – HF diet + 0.3% metformin
- Db + S A1C1 group – HF diet + 2.5% ST, 2% *L*-arg, 0.001% Cr
- Db + S A1C2 group – HF diet + 2.5% ST, 2% *L*-arg, 0.005% Cr

- Db + S A2C1 group – HF diet + 2.5% ST, 4% *L*-arg, 0.001% Cr
- Db + S A2C2 group – HF diet + 2.5% ST, 4% *L*-arg, 0.005% Cr
- Db + R A1C1 group – HF diet + 2.5% RA, 2% *L*-arg, 0.001% Cr
- Db + R A1C2 group – HF diet + 2.5% RA, 2% *L*-arg, 0.005% Cr
- Db + R A2C1 group – HF diet + 2.5% RA, 4% *L*-arg, 0.001% Cr
- Db + R A2C2 group – HF diet + 2.5% RA, 4% *L*-arg, 0.005% Cr.

Details on the test supplements, experimental protocol, chromium determination, and other analyses performed in the course of the experiment were thoroughly described in another article (Kurek et al., 2022). The experimental protocol was approved by the Local Ethics Committee in Poznań (No. 31/2019).

Trace element determinations

The livers and the kidneys were dissected free and appropriately preserved at autopsy. The samples were first pre-digested in acid (65% HNO_3, Merck KGaA, Darmstadt, Germany) and then mineralized in a microwave digestion system (Speedwave XPERT, Berghof Products + Instruments GmbH, Eningen, Germany). The concentrations of minerals in the obtained solutions were analyzed with the atomic absorption spectrometry method using an AAS-3 atomic absorption spectrometer (Carl-Zeiss AG, Jena, Germany). Certified reference materials (INCT-SBF-4, Institute of Nuclear Chemistry and Technology, Warsaw, Poland; BCR 679, Institute for Reference Materials and Measurements, Geel, Belgium; NIST1577C, NIST®, Gaithersburg, MD, USA; NCS ZC 73030, LGC Standards Ltd., Teddington, UK) were subsequently used in order to evaluate and confirm the accuracy of the method.

Statistical methods

All the obtained results were gathered and pre-calculated using Microsoft Excel 2019 (Microsoft Corporation, Redmond, USA) and analyzed using Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA). The statistical methods that were applied were: Shapiro–Wilk test, one-way analysis of variance (ANOVA), multivariate analysis of variance (MANOVA), and Fisher's Least Significant Difference post hoc test (LSD) ($p < 0.05$). All the data shown in the body of the article refer to the T2DM stage of the experiment.

RESULTS

Kidney trace element status

Tissular Cr concentrations were already described by Kurek et al. (2022), nevertheless in this work, it is important to confront this data with the concentrations of other trace minerals (Fe, Zn and Cu) in the critical organs of experimental rats.

The effects of the modified diet on kidney trace element status are presented in Tables 1 and 2.

Both induced hyperglycemia (mild diabetes) and supplementary intervention resulted in a statistically significant effect on the kidney concentrations of the analyzed trace elements. Although there was no observable difference between the C and Db groups, in terms of Fe and Cu concentrations, the administration of the tested compounds resulted in increased accumulation of these elements in the kidneys of rats in certain groups. In particular, the combination of RA, lower dose of *L*-arg and higher dose of Cr(III) or RA, higher dose of *L*-arg and lower dose of Cr(III), resulted in a significant elevation of kidney Fe levels (Db + R A1C2, Db + R A2C1) (Table 1). Higher concentrations of Cu were observed in all the supplemented groups, except for the groups receiving the lowest doses of both *L*-arg and Cr(III) (Table 1). In contrast, significant differences in kidney Zn levels were noted between the C and Db groups, as diabetic rat kidneys had higher levels of the aforementioned element, while the administration of ST combined with higher doses of *L*-arg and Cr(III) or RA, lower dose of *L*-arg, and higher dose of Cr(III) (Db + S A2C2, Db + R A1C2) normalized the content of Zn in this organ (Table 1). As expected, the concentration of Cr in the kidneys was significantly elevated in all groups receiving higher doses of this element with diets (Table 1).

The results of multivariate analysis revealed that all three experimental factors independently affected the kidney Zn levels in the following manner: rats receiving RA, a higher dose of *L*-arg, or lower dose of Cr(III) independently had higher Zn levels in the kidneys (Table 2). Moreover, the supplementary Cr level

Table 1. Results of SG, *L*-arg and Cr(III) supplementation on kidney and liver trace element status in rats

Parameter	Control, C	Db	Db + Met	Db + S A1C1	Db + S A1C2	Db + S A2C1	Db + S A2C2	Db + R A1C1	Db + R A1C2	Db + R A2C1	Db + R A2C2
						Kidney					
Fe µg/g d.m.	245.40 ±40.39^{abc}	220.92 ±18.40^{a}	251.82 ±34.96^{abc}	233.16 ±42.46^{ab}	228.96 ±21.73^{ab}	254.24 ±38.71^{abc}	227.77 ±34.24^{a}	223.55 ±35.32^{a}	271.50 ±46.22^{c}	263.45 ±51.59^{bc}	219.36 ±53.73^{a}
Zn µg/g d.m.	80.39 ±15.48^{a}	99.90 ±12.93^{de}	101.42 ±8.42^{de}	98.02 ±6.68^{cde}	96.57 ±7.48^{cde}	93.00 ±13.86^{bcd}	84.31 ±6.00^{ab}	92.05 ±7.79^{bcd}	88.22 ±10.28^{abc}	116.55 ±20.77^{f}	106.92 ±12.60^{ef}
Cu µg/g d.m.	28.11 ±7.77^{ab}	24.77 ±7.14^{a}	37.59 ±13.91^{cd}	23.01 ±5.14^{a}	35.33 ±9.26^{bcd}	38.00 ±12.75^{cd}	35.32 ±2.92^{bcd}	29.61 ±2.78^{abc}	42.50 ±13.78^{d}	36.58 ±3.40^{bcd}	35.25 ±8.63^{bcd}
Cr ng/g d.m.	1175.28 ±193.11^{a}	1165.88 ±250.83^{a}	1000.07 ±215.44^{a}	1363.77 ±281.70^{a}	4099.95 ±549.43^{c}	2489.25 ±596.15^{b}	4342.05 ±985.58^{cd}	1233.89 ±278.40^{a}	5099.20 ±987.83^{d}	1697.85 ±311.23^{ab}	3833.31 ±691.14^{c}
						Liver					
Fe µg/g d.m.	440.41 ±53.85	458.30 ±132.66	471.00 ±116.80	404.76 ±72.82	475.84 ±110.47	429.61 ±90.86	493.38 ±131.02	458.31 ±94.21	535.25 ±119.97	417.09 ±83.26	538.52 ±178.81
Zn µg/g d.m.	95.06 ±8.38	87.85 ±9.60	90.04 ±14.92	86.95 ±8.20	86.29 ±9.06	83.48 ±9.31	89.26 ±13.82	88.58 ±10.10	83.80 ±6.97	84.73 ±12.91	89.68 ±7.06
Cu µg/g d.m.	12.82 ±1.28^{ab}	12.36 ±2.11^{a}	14.52 ±2.71^{cd}	11.42 ±1.79^{a}	15.89 ±1.74^{d}	14.80 ±1.63^{cd}	14.24 ±2.19^{bc}	15.54 ±1.08^{cd}	16.01 ±1.35^{d}	15.09 ±1.48^{cd}	15.69 ±2.29^{cd}
Cr ng/g d.m.	213.79 ±74.97^{bc}	122.62 ±28.65^{a}	213.78 ±99.19^{bc}	188.83 ±45.17^{ab}	178.49 ±11.50^{ab}	200.00 ±61.72^{b}	235.00 ±91.64^{bc}	282.12 ±56.41^{c}	586.80 ±103.37^{f}	371.22 ±142.32^{d}	460.27 ±104.16^{e}

Data presented as the mean ±standard deviation.
Values with different letters (a–f) show statistically significant differences ($p < 0.05$; a < b; Fisher's LSD test).

Table 2. Main effects and interaction effects of SG, *L*-arg, and Cr(III) (multivariate analysis)

Index	Main Effects			Interaction Effects			
	Glycoside (ST vs RA)	*L*-arg (2% vs 4%)	Cr(III) (0.001% vs 0.005%)	Glycoside x *L*-arg	Glycoside x Cr(III)	*L*-argx Cr(III)	Glycoside x *L*-arg x Cr(III)
			Kidney				
Fe	235.57 ±35.26	239.29 ±40.89	243.33 ±43.97	NS	NS	**	NS
µg/g d.m.	245.11 ±50.90	241.43 ±47.12	237.35 ±43.92				
Zn	92.98 ±10.24	93.72 ±8.77	99.91 ±16.37	***	NS	NS	NS
µg/g d.m.	100.94 ±17.55**	100.19 ±18.63*	94.01 ±12.62*				
Cu	32.65 ±10.01	32.51 ±11.40	31.21 ±9.71	NS	NS	**	NS
µg/g d.m.	36.34 ±9.81	36.26 ±8.04	37.10 ±9.59*				
Cr	3 073.75 ±1383.47	2 949.20 ±1804.11	1 696.19 ±620.46	***	*	***	NS
ng/g d.m.	2 966.06 ±1707.92	3 090.62 ±1253.74	4 343.63 ±926.08***				
			Liver				
Fe	451.44 ±106.09	466.83 ±106.97	427.39 ±84.58	NS	NS	NS	NS
µg/g d.m.	483.23 ±126.93	467.01 ±128.25	508.58 ±132.28**				
Zn	86.57 ±10.15	86.47 ±8.52	80.00 ±10.08	NS	NS	NS	NS
µg/g d.m.	86.61 ±9.68	86.72 ±11.22	87.22 ±9.72				
Cu	14.07 ±2.46	14.66 ±2.46	14.16 ±2.23	NS	NS	**	**
µg/g d.m.	15.56 ±1.55***	14.92 ±1.90	15.43 ±1.98**				
Cr	202.37 ±63.58	319.65 ±181.23	260.54 ±110.80	NS	***	*	**
ng/g d.m.	425.10 ±152.93***	316.62 ±145.76	380.27 ±186.85***				

Data presented as the mean ±standard deviation.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (statistically significant differences).

influenced the kidney Cu and Cr content, as rats receiving higher doses of Cr(III) had significantly higher levels of Cu and Cr in this organ. Several interaction effects between experimental factors on some trace element levels in the kidneys were also observed: the lowest kidney Fe concentrations were found when either lower or higher doses of *L*-arg and Cr(III) were combined. The interaction between RA and a higher dose of *L*-arg was the most significant in increasing kidney Zn levels. The kidney Cu concentration was significantly lower when lower doses of both *L*-arg and Cr(III) were combined. A higher concentration of Cr(III) in a diet combined with either RA or a lower dose of *L*-arg resulted in the most increased accumulation of this element in the tissue. Surprisingly, however, a similar effect was observed in the case of a combination of ST and a higher dose of *L*-arg.

Liver trace element status

The effects of the modified diet on the liver trace elements status are presented in Tables 1 and 2.

The effects of supplementary intervention on liver trace element content were visibly weaker compared to the effects observed in the kidneys. Hyperglycemia did not affect liver trace element levels, except for Cr, where its content was significantly lower in the Db group vs. the healthy C group (Table 1). Of the aforementioned trace elements, the tested supplementary compounds affected only liver Cu content. Groups receiving the tested agent combinations (except for ST + lower doses of both *L*-arg and Cr(III)) had significantly higher levels of the aforementioned element in the liver, while the Fe and Zn concentrations were kept unchanged (Table 1). Liver Cr level, however, was significantly lower in the Db group compared to the healthy control. Supplementary intervention tended to alleviate the disturbance, normalizing or even exceeding tissular Cr levels in certain groups, especially those receiving a higher dose of the element with the diet (Db + S A2C2, Db + R A1C2, Db + R A2C2).

The results of multivariate analysis showed that two of the experimental factors (the type of SG and the level of Cr) independently affected trace element levels in the liver. In particular, liver Fe content was significantly higher in rats receiving higher doses of Cr(III) (Table 2). Liver Cu and Cr concentrations were markedly higher in groups receiving RA and/or Cr(III) in higher doses independently. A few interaction effects were also observed. This time, the interaction between RA and a higher dose of Cr(III) was the most pronounced and resulted in the highest concentration of Cr in the liver. This effect was even amplified when the aforementioned factors were put together along with the lower dose of *L*-arg. However, the lowest liver Cu level was observed when the combination of ST and lower dose of *L*-arg was applied. The aforementioned combination in conjunction with a lower dose of Cr(III) resulted in the most substantial decrease.

DISCUSSION

In general, the evaluation of trace element (Fe, Zn, Cu, and Cr) status in animals and humans is a challenging task, due to the variety of functions performed by particular elements in physiology and metabolism, as well as differences in the distribution and storage of individual minerals in the body. Usually, trace element status is evaluated based on measuring its bioactive form concentrations in selected fluids and tissues; the more types of samples analyzed, the more precise the assessment can be. For example, Fe status can be evaluated based on the concentration of specific Fe-containing molecules in the blood (blood hemoglobin-related indices), as well as its storage pools (e.g. blood ferritin, liver Fe content), while Cr status is difficult to assess, due to lack of reliable biomolecules and its fast turnover in the body. In this study, trace element (Fe, Zn, Cu, and Cr) status was evaluated based on the storage pool of these elements, expressed by their total concentrations in the liver and kidneys of rats.

From the perspective of carbohydrate metabolism, micronutrients are necessary to enable certain functions in the human body. For instance, they enhance the insulin pathway by activating insulin receptor binding sites, they act as components for enzymes crucial for glucose metabolism, and/or improve insulin sensitivity, among others. What is more, it has been found that prolonged hyperglycemia can lead to severe abnormalities in trace element status in the body (Mooradian et al., 1994). With the homeostasis of micronutrient status disrupted due to diabetes, there is an increase in metabolic demand. Data specifically on trace element levels abnormalities in tissues are limited; however, data on the link between in blood serum mineral status

and metabolic status are fairly abundant. Although the relation between Fe body levels and carbohydrate metabolism is not fully understood, the results of experimental studies show that variations of this element can be indicators of diabetes development and a number of epidemiological studies describe the correlation of Fe status and circulating ferritin levels with T2DM development (Krisai et al., 2016). A study performed by Lao et al. (2001) revealed a higher risk of developing gestational diabetes mellitus in pregnant women with elevated concentrations of serum ferritin, Fe, and transferrin saturation even with no observable change in body mass or hemoglobin levels in the third trimester (Lao et al., 2001). What is more, it was found that the risk of T2DM development can correlate with changes in ferritin levels (independently of other known risk factors) (Jiang, 2004). Also, Fe, as a pro-oxidant, elevates oxidative stress and thus can increase the risk of T2DM (Dongiovanni et al., 2013). Zn is an important element in cell signaling and various cellular processes associated with carbohydrate metabolism and therefore diabetes and insulin sensitivity (Dubey et al., 2020). T2DM subjects have been found to have decreased serum Zn concentrations (Kumar et al., 2014). The effect may be caused by an increased urinary Zn loss in the course of T2DM (Bandeira et al., 2017) or disturbances of Zn reabsorption in the kidneys due to hyperglycemia (Puri et al., 2013). However, there are also reports stating no link between serum Zn concentrations and glycemic control (Estakhri et al., 2011). Cu has been shown to participate in insulin receptor activation and thus enhance insulin activity (Dubey et al., 2020). Experimental studies show that T2DM patients have higher copper levels (Kumar et al., 2014; Viktorínová et al., 2009). Also, levels of serum Cu are linked to HbA1c concentrations in T2DM patients (Xu et al., 2013). On the other hand, other experiments revealed that diabetic children had lower concentrations of serum Cu than controls (Ahmed and Helal, 2002). Many experimental studies and clinical trials have reported that Cr status (e.g. in humans indicated by lower serum Cr level or increased urine excretion, while in animals by lower tissular Cr levels) is usually lowered in hyperglycemia and chronic T2DM (Havel, 2004). Disrupted Cr body levels can lead to the development of symptoms related to diabetes and/or cardiovascular diseases (Dubey et al., 2020). Cr has been shown to improve glucose and insulin levels in hypo- and hyperglycemic patients with no noticeable side effects in controls. This element is known to increase insulin sensitivity, i.e. by improving insulin binding and β-cell sensitivity (Anderson, 1997). However, the mechanisms of these phenomena are not entirely clear.

The results of the present study show that the only changes in the status of the trace elements observed were an increase in kidney Zn level and a decrease in liver Cr level. This is most likely due to the severity of the disease, i.e. the early, mild stage of T2DM. Potential implications of the observed alterations on the overall metabolic function are as follows: disruption of critical metalloenzymes by competitively inhibiting enzymes that require other metal cofactors; impairment of detoxification processes; elevation of oxidative stress within renal tissues; imbalances in electrolytes (sodium, potassium, and calcium) affecting processes such as muscle contraction, nerve function, and fluid balance; exacerbating insulin resistance in type 2 diabetes by disruptions in insulin storage and secretion in the pancreas (Vallee and Falchuk, 1993) and overall impairment of insulin function and glucose metabolism (Mertz, 1993). The dietary co-supplementation using three experimental factors, such as the combination of certain SG (ST or RA), *L*-arg (2% or 4%), and/or Cr(III) (0.001% or 0.005%) can significantly affect or, in some specific cases, regulate the trace element status of type 2 diabetic rats.

The majority of studies published in the literature were focused more on the effects of various fractions or extracts of stevia than its pure compounds. In the literature, information about the effects of SG on body trace element status is lacking, especially regarding disturbances caused by diabetes. The only available work referring to this topic is conference material by Kurek and Krejpcio (2022). These authors concluded that supplementary SG (ST, RA) did not affect liver trace elements status in severe T2DM. Both ST and RA showed only a slight tendency to increase kidney Fe and Cu concentrations. The present study showed for the first time that, independently of other experimental factors, the type of SG (ST, RA) affects the content of Zn, Cu, and Cr in internal organs. RA was more capable of increasing the content of Cu and Cr in the liver and Zn in the kidney of mild diabetic rats. The mechanism responsible for this effect is unclear.

In light of this, it can be hypothesized that supplementary SG (ST, RA) have an impact on overall metabolism that in turn involves alteration of Zn, Cu, and Cr in the storage pools of these elements in the course of hyperglycemia.

The second experimental factor that independently affected some trace element levels in this study was the dose of supplementary *L*-arg. Although the dose of the agent did not significantly change trace element levels in the liver or kidneys, its administration in a higher dose significantly increased kidney Zn content in diabetic rats. *L*-arg dose in the animals' diets was not crucial for mineral composition in most cases; however, a higher dose of the amino acid led to a significant increase of Zn in the kidney. The mechanisms underlying these effects are not clear. *L*-arg is a known precursor of nitric oxide synthesis and a crucial factor for several physiological processes. Elevated nitric oxide concentration has been reported to induce Zn release in endothelial cells and to intensify metallothionein expression (Li et al., 2010; Wiseman et al., 2006). Metallothioneins are proteins that store Zn, thus they are involved in the homeostasis of the element (Formigari et al., 2007). Zn is also an essential co-factor for the function of SOD (Cu/Zn superoxide dismutase), which is a protein being stimulated during oxidative stress and catalyzing the process of dismutation of superoxide anion $^{\cdot}O_2^-$ to H_2O_2 (Fukai and Ushio-Fukai, 2011). *L*-arg has protective properties against ROS (reactive oxygen species) through chemical interaction with oxygen molecules and therefore improves the overall antioxidant status (Lass et al., 2002). Zn and *L*-arg were shown to synergistically affect glutathione metabolism (Bergeron and Guay, 2019). The mechanism underlying the effect has not been fully explained; however, it has been proposed that the amino acid could induce the effect by stimulating the synthesis of nitric oxide and through Zn release from MT1 protein (Poeze et al., 2011; Wiseman et al., 2006). Zn supplementation was shown to increase Zn concentrations in plasma; however, it is worth noticing that the effect was intensified by additional *L*-arg supplementation, which could suggest an improvement in Zn release from tissues and/or intestinal Zn absorption (Bergeron and Guay, 2019; Walk et al., 2015).

The third experimental factor that independently affected some trace element levels in this study was the dose of supplementary Cr(III). Cr status is usually decreased in hyperglycemia and chronic T2DM. Most studies investigating Cr body levels in diabetes showed that the disease can lead to a significant decrease in this element (Anderson, 1998; Chen et al., 2022; Dubey et al., 2020; Rajendran, 2015; Sundararaman et al., 2012), which suggests an increase of its loss due to the hyperglycemia-related metabolic stress. Regarding supplementary Cr(III), especially when administered at a higher dose, as expected, it significantly elevated the depleted Cr stored in the liver and kidneys of diabetic rats. Furthermore, it also markedly increased liver Fe and Cu content, as well as the kidney Cu, but decreased Zn levels in diabetic rats, which confirms that these trace elements (Fe, Zn, Cu) are co-dependent on the absorption, transporting, distribution, metabolic, and excreting stages.

In the presented study, multiple interactions of experimental factors that affected certain trace element levels in the liver and kidneys as a result of co-supplementation were observed. Liver Cu levels were significantly elevated in most diet-treated rats, compared to the control and diabetic untreated animals. The reason for the aforementioned shift is unclear. However, it could be reasonably hypothesized that the metabolism of SG, particularly RA, combined with supplementary *L*-arg and Cr, triggers the shift of Cu from other storage tissues to the liver, where it plays a role in processing these agents more efficiently. However, this assumption needs to be confirmed in further study due to the complexity of the mechanisms of interactions between individual factors.

CONCLUSIONS

The results of this study make it possible to conclude that even mild hyperglycemia in type 2 diabetes disturbs the balance of trace elements (Zn, Cr) by shifting their concentrations in the critical organs (liver, kidneys) in rats. Supplementary agents (ST, RA, *L*-arg, Cr(III)) used independently, or in certain combinations, can mitigate some trace element alterations or even cause further changes in their concentrations in the liver or the kidneys. The metabolic significance of these alterations is not fully understood and warrants further studies, especially human trials, in order to better understand and explore the described mechanisms.

LIMITATIONS

The main limitation of the study is the relatively small number of animals in the groups ($n = 10$). This is due to the conditions and requirements of the guidelines of the Declaration of Helsinki and the Local Ethics Committee. It should be acknowledged that the present experiment refers to an animal model. They should be interpreted with caution, as the results may differ in humans.

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DECLARATIONS

Data statement
All data supporting this study has been included in this manuscript.

Ethical Approval
Not applicable.

Competing Interests
The authors declare that they have no conflicts of interest.

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