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Evidence for safety of the dietary ingredient agmatine sulfate as assessed by mutagenicity and genotoxicity studies

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ABSTRACT

Agmatine, 1-Amino-4-guanidinobutane, is a ubiquitous naturally occurring molecule present in low amounts in a wide variety of foodstuff. Clinical trials have demonstrated the safety of oral agmatine sulfate and have led to its development as an effective dietary ingredient for promoting resilient nerve functions. Although clearly required, the mutagenic and genotoxic effects of agmatine have not been previously reported. The present study, therefore, undertook to assess the safety profile of agmatine using currently accepted *in vitro* and *in vivo* mutagenicity and genotoxicity tests. The test item was G-Agmatine®, a proprietary brand of agmatine sulfate. Using the bacterial reverse mutation assay (Ames test), the study found that G-Agmatine® has no mutagenic effects. It had no clastogenic effects as observed by the *in vitro* chromosomal aberration test using Chinese Hamster lung cells. And it lacked genotoxic effects as evidenced by the lack of increased frequency of micronucleated polychromatic immature erythrocytes following oral administration in the mouse micronucleus test. Taken together with previously published data, results of the present study further support the safety of agmatine sulfate as a dietary ingredient.

1. Introduction

Agmatine is a naturally occurring metabolite of the amino acid arginine. Based on the discovery of agmatine's neuroprotective properties [10], which has been substantiated by extensive worldwide scientific research [15,36], the sulfate salt of agmatine has been developed as a dietary ingredient and was introduced to market in dietary supplement products [17,27].

Agmatine, 1-Amino-4-guanidinobutane decarboxylated arginine ($NH_2(CH_2)_4NH_2C(NH{=})NH$], sometime termed decarboxylated arginine, is a ubiquitous molecule in living organisms, discovered a century ago by Albrecht Kossel (1910) [14]. Substantial preclinical and initial clinical evidence suggests the utility of agmatine in treating a wide spectrum of complex diseases involving various bodily systems [24,25]. These include nervous system conditions such as: neurotrauma (e.g., stroke, brain and spinal cord injury, glaucoma), neuropathies and neuropathic pain, opioid analgesia and addiction, neurodegenerative diseases (e.g., Parkinson's disease) and mood (e.g., anxiety, depression) and cognitive disorders (e.g., Alzheimer's disease); and conditions involving the kidneys, cardiovascular system, gastrointestinal (GI) system, liver, and glucose metabolism.

Presently, however, there are no health-related conditions other than neuropathies and related symptoms for which agmatine treatment had demonstrated effectiveness in human [17,27] and companion animals [32,33] clinical studies. Noticeably, touting agmatine sulfate-containing products for bodybuilding or muscle building sports is completely unsubstantiated and is hyped by outright false claims.

It is postulated that agmatine exerts its beneficial actions by modulating, potentially synergistically, multiple molecular targets and hence, we metaphorically refer to it as a 'molecular shotgun'. These targets were summarized by Piletz et al. [24] and include: (A) several neurotransmitter receptors and receptor ionophores; (B) key ionic channels

Abbreviations: 9AA, 9-Aminoacridine; 2AA, 2-aminoanthracene; AGE, advanced glycation end products; CP, Cyclophosphamide monohydrate; DME, Dulbecco's Modified Eagle's (medium; EMS, Ethyl Methanesulphonate; GABA, γ-aminobutyric acid; GI, gastrointestinal; GLP, good laboratory practice; HPLC, high-performance liquid chromatography; MMPs, matrix metalloproteases; MMS, Methyl methanesulfonate; MPCE, micronucleated polychromatic erythrocytes; MR, Mutation rate; NCE, normochromatic erythrocytes; NO, nitric oxide; NPD, 4-Nitro-1,2-phenylenediamine; OECD, Organization for Economic Co-operation and Development; PCE, polychromatic erythrocytes; Revs, revertants; SAZ, sodium azide; SPF, specific-pathogen-free.

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and membrane transporters; (C) nitric oxide (NO) formation; (D) polyamine metabolism; (E) protein ADP-ribosylation and hence signaling pathways; (F) matrix metalloproteases (MMPs), enzymes implicated in nerve cell death and neuropathic pain; and (G) advanced glycation end (AGE)-product formation, a process involved in the pathology of diabetes and neurodegenerative diseases.

While agmatine is synthesized in the body [18], it is also acquired from diet where it is found in low amounts in a wide variety of plant-, fish- and animal-derived foodstuff [19. Additionally, many GI bacteria produce agmatine [6], and the high concentrations of agmatine found in the mammalian GI tract implicate microbial production as the main source of systemic agmatine [12,19,31]. Animal studies have demonstrated that exogenous agmatine sulfate, the commonly used salt of agmatine, is absorbed in the GI tract and then rapidly (within minutes) distributed throughout the body [19], including the brain [24]. In humans, ingested agmatine is readily absorbed and eliminated unmetabolized by the kidneys with an apparent blood half-life of about 2 hours [13].

Agmatine is principally metabolized into urea and putrescine [11], the diamine precursor of polyamines, molecules which are not only essential for cell proliferation, but also for viability of mature cells in general and specifically so for nerve cells [9,10]. Agmatine-derived putrescine may also serve as a minor precursor for the neurotransmitter GABA (γ-aminobutyric acid) [19]. Additionally, agmatine can also be oxidized, resulting in agmatine-aldehyde formation, which may be toxic and secreted by the kidneys [28]. Yet agmatine aldehyde by suppressing inducible nitric oxide synthase mediated nitric oxide generation, can be protective in a model of endotoxic shock (i.e., sepsis) [28]. This latter metabolic route is probably tissue specific, being significant in some tissues [19], but minor in others [5,32] and apparently negligible in the central nervous system [19].

The intense interest in agmatine's therapeutic potential [24], has clearly indicated the requirement for assessing the longer-term effects of oral agmatine treatment. Observations in laboratory rats have provided evidence that sub-chronic (95 days) high dosage oral agmatine sulfate regimen (829.85 and 568.51 mg/kg per day in female and male rats, respectively) is safe [7]. A study with adult mice demonstrated the lack of adverse effects by oral agmatine sulfate during both 7-day treatment with 900 mg/kg per day, and 95-day treatment with 300 mg/kg per day, and gross necropsy and organ histology revealed no pathological alterations after the 95-day treatment [4]. Moreover, no adverse effects were observed in clinical studies performed with dogs [32] (28-day oral treatment with 50 mg/kg per day) and horses [33] (30-day oral treatment with 25 mg/kg per day). As previously mentioned, human clinical trials by Keynan et al. [17] have shown that a regimen of high doses oral agmatine sulfate (up to 3.560 g/day) given for up to 3 weeks, is safe. And a long-term case report indicated that intake of 2.67 g/day for 5 years has no measurable unwanted effects [8].

In vitro studies have shown that agmatine exerts differential effects on the proliferative capacity of various cell types. Thus, while agmatine is long known to stimulate proliferation of thymocytes and lymphocytes [34,35], and, more recently, of neural stem cells [16,30], it can also exert rather anti-proliferative effects on various other cell types including smooth muscle cells, macrophages, fibroblasts, astrocytes, as well as cancer cells [10,16,20,26,30]. Apparently, the effects of agmatine on cell proliferation depend on cell type and on the stage of cellular differentiation. Importantly, while agmatine is considered cytotactic for certain cell types, it is however, not cytotoxic [11,20].

Summarized data from high-throughput screening assays, show that out of 119 screened targets, agmatine sulfate is inactive in practically all of them, including CYP3A4 (cytochrome P 450 3A4 isoform 1), with few inconclusive results and only 1 active (i.e., binding at the multidrug-resistance transporter) [1]. These data thus, further indicate that agmatine sulfate is not likely to exert non-specific cytotoxic side effects, and furthermore, that agmatine is probably not involved in interactions with other drugs.

Although clearly required, standardized studies to assess whether agmatine exerts any mutagenic or genotoxic effects have not been reported thus far. In view of this paucity, therefore, the objective of the present study was to ascertain the safety profile of agmatine sulfate using a battery of generally accepted *in vitro* and *in vivo* mutagenicity and genotoxicity tests.

2. Materials and Methods

2.1. Principle of the Study

The test item was G-Agmatine®, a proprietary brand of agmatine sulfate, $H_2N(CH_2)_4NHC(=NH)NH_2 \cdot H_2SO_4$ (CAS No. 2482–00–0). There are three basic methods for synthesizing agmatine sulfate: chemical synthesis, using precursor chemicals; fermentation biosynthesis, using bacteria growing in appropriate culture medium; and biochemical enzymatic synthesis, using partially purified bacterial agmatine decarboxylase. All three methods yield an equally pure, high-quality product. For the present study, agmatine sulfate was synthesized by the fermentation method for Gilad&Gilad LLC (Las Vegas, NV 89129, USA), in compliance with the Food Safety Management System standard GB/T 22000–2006/ISO22000:2005. Production by the fermentation biosynthetic method consists of the following major steps: culturing non-genetically modified Brevibacterium spp in a fermentation medium in the presence of L-Arginine and H_2SO_4, followed by microfiltration, ion exchange separation, and crystallization steps. The biosynthesized agmatine sulfate was analyzed by a high-performance liquid chromatography (HPLC) method to ascertain identity and purity, and its physical properties were characterized. The preparation was tested to exclude the presence of heavy metals, yeast, and bacteria, and was issued with a certificate of analysis (COA).

Prior to manufacturing agmatine sulfate-containing products, the procured raw material (agmatine sulfate) was analyzed again using a HPLC method to ascertain identity and purity and re-tested for heavy metals and microbiology to ensure compliance with the highest standards required of dietary ingredients. For product manufacturing, the G-Agmatine® brand of agmatine sulfate, was encapsulated as the sole ingredient, without any excipients, in hard-shell gelatin capsules.

The final product, G-Agmatine®-containing capsules, was re-analyzed using the same battery of tests and issued with a product COA. For the present battery of toxicity tests, Gilad&Gilad LLC has provided a sample of G-Agmatine® as the test article, along with a product COA, and material safety data sheet.

G-Agmatine® safety was assessed according to the most common approach for a Good Laboratory Practice (GLP) test battery in the form of 2 in vitro assays (a bacterial reverse mutation and a mammalian cell tests) and one in vivo assay [3], which were conducted consecutively. The bacterial reverse mutation assay (Ames test) was performed first, followed by the *in vitro* mammalian chromosomal aberration assay and thereafter, by the *in vivo* mouse micronucleus assay. All assays and animal studies were conducted in accordance with GLP regulations at Toxi-Coop laboratories, a facility certified by the Hungarian Ministry of Human Capacities and by the Hungarian Ministry of Agriculture and Regional Development. All chemicals used in the experiments were of analytical grade. Chemicals, solutions, and nutrient mixtures were supplier/manufacturer-certified and were used prior to their indicated expiry dates.

2.2. Bacterial Reverse Mutation Assay (Ames test)

The mutagenicity of agmatine sulfate (G-Agmatine® brand) was assessed in bacteria by the Ames test [2] in accordance with the Organization for Economic Co-operation and Development (OECD) 471 guidelines [22]. Experiments were carried out using histidine-requiring auxotroph strains of *Salmonella typhimurium* (*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537), and the tryptophan-requiring

Table 1
Mammalian Chromosomal Aberration experiments, indicating the concentrations and exposure/expression intervals of G-Agmatine® (agmatine sulfate) in the presence or absence of S9 activation system.

G-Agmatine® Concentrations (ug/ml)	Exposure/Expression Intervals (hr)	S9 Activation System
Experiment A		
500, 1000, 2000	3/20	With and Without S9
Experiment B		
B.I - 375, 750, 1500	20/20	Without S9
B.II - 375, 750, 1500	20/28	Without S9
B.III - 500, 1000, 2000	3/28	With S9

auxotroph strain of *Escherichia coli* (*E. coli* WP2 *uvrA*) (supplier: Trinova Biochem GmbH, D-35394 Giessen, Germany; manufacturer: MOLTOX INC., BOONE, NC 28607 USA). Tests were conducted in the absence and presence of post mitochondrial supernatant metabolic activation system (S9) prepared from phenobarbital/β-naphthoflavone-induced rat livers [2,22]. The study included a Preliminary Solubility Test, a Preliminary Concentration Range Finding Test (Informatory Toxicity Test), an Initial Mutation Test (Plate Incorporation Test), and a Confirmatory Mutation Test (Pre-Incubation Test). Based on the results of the Solubility and Concentration Range Finding Tests, G-Agmatine® was dissolved in ultrapure (ASTM Type 1) water (as a vehicle compatible with bacterial survival) and its effects were assessed in the absence and presence of S9 activation system. Additionally, appropriate historical control data (testing laboratory's own database) were available for comparison. Based on the preliminary tests, the following concentrations of the test item (G-Agmatine®) were investigated in the Initial and Confirmatory Mutation Tests: 16, 50, 160, 500, 1600, and 5000 μg/plate. Tests were performed in triplicate measurements. For the *S. typhimurium* TA1535 and TA1537 strains, the criterion for classifying a substance as mutagenic was an increase in the mean number of revertants of at least 3 times greater than the observed negative control means, and 2 times greater than controls in the other three strains.

2.3. In Vitro Mammalian Chromosomal Aberration Assay

To assess whether the test item, agmatine sulfate (G-Agmatine® brand), has genotoxic effects, the *in vitro* chromosome aberration assay was conducted in accordance with OECD Guideline No. 473 [23]. The assay was conducted in the absence and presence of S9 activation system. Experiments were conducted using Chinese hamster lung (male) cells [V79 cell line, supplier: European Collection of Cells Cultures (ECACC)]. Cells were seeded into 92 ×17 mm culture dishes at 5 $\times 10^5$ cells each and were incubated for 24 hours in 10 ml of DME (Dulbecco's Modified Eagle's) medium containing 10 % fetal bovine serum.

Agmatine sulfate was dissolved in DME medium, and cells were exposed to a dose range based on the following: 1) the maximum recommended concentration of 2000 μg/ml for soluble, lower cytotoxic substances [23]; 2) cytotoxicity investigations made in a preliminary study (with or without metabolic S9 activation system). Ethylmethane sulphonate (EMS) treatment (0.4 and 1.0 μl/ml), a known mutagen and clastogen, served as positive control for assays performed in the absence of S9 activation system. Cyclophosphamide monohydrate (CP) treatment (5.0 μg/ml) served as positive control for assays in the presence of S9 activation. The solvent, DME medium, served as negative control. In two independent experiments (both run in duplicate with concurrent negative and positive controls), at least 150–150 well-spread metaphase cells were analysed at concentrations and at exposure/expression intervals (i.e., treatment and sampling times) as outlined in Table 1.

Following different incubation intervals, cells were exposed to the selection agent Colchicine (0.2 μg/ml) for 2.5 hours prior to harvesting. Harvested cells were fixed, placed on slides, and stained with Giemsa stain for light microscopy detection of chromosome aberration in cells at metaphase. Structural chromosome aberrations were analyzed, and their frequency scored for at least 150–150 well-spread metaphase cells.

2.4. In Vivo Mouse Micronucleus Assay

The mouse micronucleus test is another important assay for testing chromosomal aberration [29]. The potential mutagenic activity of agmatine sulfate (G-Agmatine® brand) was examined in bone marrow of 8-week-old female (26.3 – 31.0 g) and male (34.3 – 40.9 g), specific-pathogen-free (SPF), NMRI BR mice (Toxi-Coop Zrt., Budapest, Hungary). The NMRI BR mouse is one of the accepted strains for the micronucleus test. Same sex animals were kept 2–5 to a cage under standard conditions of temperature (22±3^0 C), humidity (40–70 %), and light (12 h light-dark cycle, lights on at 06:00 AM) with a free supply of food (pellet rat chow) and water in Toxi-Coop Zrt. accredited vivarium, in accordance with the Institutional Animal Care and Use Committee (Toxi-Coop Zrt., laboratories) approved protocols. Altogether, 39 females and 39 males were randomly assigned to control and test groups. The pre-test assay was conducted with a group of 2 males and 2 females to identify the appropriate maximum dose level for the main test. The test itself was carried out with 5 animals each in the low and middle doses and their corresponding positive control groups, and 10 animals in the high dose and its control group. Additional 2 males and 2 females were treated with the highest dose as a back-up group, but eventually were not used for sampling.

G-Agmatine® was dissolved in water (Aqua ad injectabilia) and administered in a constant volume (10 ml/kg body weight) by oral gavage. The test doses of G-Agmatine® were determined following a preliminary (pre-test) 48-hour toxicity study, which showed no adverse reactions or toxic signs following a single 2000 mg/kg treatment, the maximum dose level (results not shown). The final selected doses were 500, 1000 and 2000 mg/kg. Negative (vehicle) control and a positive control group were included. G-Agmatine® and negative control solutions were administered as a single oral dose. Cyclophosphamide (60 mg/kg) served as positive control; it was dissolved in physiological saline and administered once intraperitoneally (ip) in a volume of 10 ml/kg. In the low and mid dose groups, sampling from bone marrow was performed once, at 24 hours after treatment. In the high dose and corresponding negative control groups, sampling was done twice: at 24 and 48 hours after treatment. In animals treated with Cyclophosphamide, sampling was performed only once, at 24 hours post-treatment.

Bone marrow smears were prepared on microscope slides from each treatment group in accordance with OECD Guideline No. 474 [21]. Following methanol fixation, the smears were air dried at room temperature, stained with Giemsa (10 %) solution for 25 min, rinsed in distilled water, air dried, coated with EZ-mount and examined by light microscopy. The numbers of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) were scored per animal to assess the micronucleated cells. The frequency of micronucleated cells (ratio of PCE/NCE) was expressed based on the first 4000 counted PCEs.

2.5. Statistical Analysis

Results were calculated and presented by descriptive statistics as the mean ± standard deviation (SD) values of three separate measurements. Significance of differences between revertant colonies in the Ames assay, was evaluated by independent two-sample *t*-test. For the chromosomal aberration assays, the chi-squared test was performed to analyze the significance of changes in the number of aberrant cells. Assessments of the significance of differences in the numbers and frequencies of micronucleated polychromatic erythrocytes in the micronucleus assays, was performed using Kruskall-Wallis non-parametric analysis of variance (ANOVA) test.

Table 2
Effect of agmatine sulfate (as G-Agmatine® brand) on revertant colonies in the Ames Initial Mutation Test.[†].

Concentrations (μg/plate)	*Salmonella typhimurium* tester strains																*Escherichia coli*			
	TA 98				WP2uvrA				TA 1535				TA 1537				WP2uvrA			
	-S9		+S9		-S9		+S9		-S9		+S9		-S9		+S9		-S9		+S9	
	Revs	MR	Revs	MR	Revs	MR	Revs	MR	Revs	MR	Revs	MR	Revs	MR	Revs	MR	Revs	MR	Revs	MR
Solvent (Negative) Control																				
Untreated Control	23.0 ±4.4	1.28	24.0 ±6.1	1.06	95.0 ±3.5	1.14	102.7 ±2.1	0.91	10.0 ±3.6	1.20	9.7 ±1.2	1.26	11.0 ±2.6	1.03	10.7 ±3.5	1.07	22.3 ±1.5	1.31	22.3 ±3.2	0.85
DMSO Control	20.7 ±1.2	1.00	21.0 1.0	1.00	–	–	95.0 ±4.4	1.00	–	–	6.7 ±2.1	1.00	9.3 ±1.2	1.00	9.3 ±3.5	1.00	–	–	21.3 ±2.3	1.00
Ultrapure Water Control	18.0 ±5.3	1.00	22.7 ±8.6	1.00	83.3 ±1.5	1.00	113.0 ±6.0	1.00	8.3 ±2.3	1.00	7.7 ±2.1	1.00	10.7 ±2.1	1.00	10.0 ±4.6	1.00	17.0 ±1.0	1.00	26.3 ±2.1	1.00
Agmatine Sulfate (G-Agmatine®) μg/plate																				
16	23.3 ±11.4	1.30	21.7 ±5.5	0.96	97.7 ±9.1	1.17	109.0 ±4.6	0.96	9.0 ±4.6	1.08	7.0 ±3.6	0.91	9.7 ±1.2	0.91	10.3 ±5.8	1.03	17.7 ±4.0	1.04	23.7 ±4.0	0.90
50	19.0 ±2.6	1.06	26.7 ±3.5	1.18	93.3 ±16.2	1.12	99.7 ±9.0	0.88	9.3 ±2.9	1.12	7.7 ±3.2	1.00	11.3 ±3.2	1.06	10.7 ±3.2	1.07	19.7 ±5.5	1.16	27.7 ±2.5	1.05
160	21.0 ±4.6	1.17	22.0 ±1.0	0.97	91.3 ±9.9	1.10	103.0 ±12.2	0.91	11.3 ±2.9	1.36	7.0 ±5.6	0.91	10.0 ±2.0	0.94	11.7 ±3.5	1.17	25.0 ±3.6	1.47	23.3 ±2.1	0.89
500	19.3 ±4.2	1.07	24.3 ±4.2	1.07	91.3 ±18.0	1.10	108.3 ±8.0	0.96	7.3 ±3.5	0.88	10.3 ±0.6	1.35	11.0 ±4.6	1.03	8.0 ±3.6	0.80	21.0 ±4.6	1.24	24.3 ±1.5	0.92
1600	19.3 ±0.6	1.07	19.7 ±3.5	0.87	94.0 ±4.0	1.13	89.7 ±7.8	0.79	9.3 ±3.5	1.12	9.0 ±1.7	1.17	10.0 ±2.6	0.94	12.7 ±3.5	1.27	19.7 ±1.5	1.16	24.7 ±2.1	0.94
5000	22.0 ±9.8	1.22	21.7 ±2.5	0.96	104.0 ±5.3	1.25	106.0 ±7.8	0.94	7.7 ±0.6	0.92	11.0 ±4.6	1.43	11.3 ±2.5	1.06	9.3 ±5.1	0.93	23.0 ±2.6	1.35	25.3 ±2.1	0.96
Positive Control																				
NPD (4 μg)	316.7[a] ±21.4	15.32	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SAZ (2 μg)	–	–	–	–	833.3[a] ±98.7	10.00	–	–	767.3[a] ±106.0	92.08	–	–	–	–	–	–	–	–	–	–
9AA (50 μg)	–	–	–	–	–	–	–	–	–	–	–	–	428.0[a] ±49.6	45.86	–	–	–	–	–	–
MMS (2 μL)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	537.3[a] ±105.5	31.61	–	–
2AA (2 μg)	–	–	820.0[a] ±281.2	39.05	–	–	1164.7[a] ±66.5	12.26	–	–	126.7[a] ±7.4	19.00	–	–	174.3[a] ±13.3	18.68	–	–	–	–
2AA (50 μg)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	145.3[a] ±4.5	6.81

†Bacteria test strains grown in the absence (-) or presence (+) of S9 activation system, were exposed to escalating agmatine sulfate (as G-Agmatine®) concentrations along with appropriate solvent (negative) controls and positive controls and incubated at 37^0 C for 48 hr. Ultrapure water was the solvent of G-Agmatine® and of the positive control substances: SAZ (sodium azide) and MMS (Methyl methanesulfonate). DMSO (Dimethyl sulfoxide) was the solvent of the positive control substances: NPD (4-Nitro-1,2-phenylenediamine), 9AA (9-Aminoacridine) and 2AA (2-aminoanthracene). Mutation rate (MR) values of colonies treated with G-Agmatine®, SAZ, MMS and of untreated control were calculated in comparison to ultrapure water solvent values. MR values of NPD, 9AA and 2AA were calculated in comparison to DMSO solvent values. Results are expressed as mean revertants (Revs) per plate ±SD values, and as MR values, of three experiments. [a]$p < 0.05$ as compared to the relevant negative controls.

Table 3
Effect of agmatine sulfate (as G-Agmatine® brand) on revertant colonies in the Ames Confirmatory Mutation Test.[†].

Concentrations	*Salmonella typhimurium* tester strains																*Escherichia coli*			
	TA 98				TA 100				TA 1535				TA 1537				WP2uvrA			
	-S9		+S9		-S9		+S9		-S9		+S9		-S9		+S9		-S9		+S9	
	Revs	MR	Revs	MR	Revs	MR	Revs	MR	Revs	MR	Revs	MR	Revs	MR	Revs	MR	Revs	MR	Revs	MR
Solvent (Negative) Control																				
Untreated Control	22.3 ±4.2	1.81	25.0 ±5.6	0.84	88.3 ±2.1	0.93	117.0 ±13.9	0.96	12.7 ±2.3	1.15	11.7 ±2.1	1.03	8.0 ±2.7	0.71	10.0 ±2.0	1.15	18.0 ±6.1	0.77	26.0 ±3.5	1.05
DMSO Control	17.3 ±2.1	1.00	22.7 ±3.1	1.00	–	–	107.7 ±8.1	1.00	–	–	10.0 ±1.7	1.00	8.7 ±5.0	1.00	7.7 ±0.6	1.00	–	–	21.0 ±6.1	1.00
Ultrapure Water Control	12.3 ±3.2	1.00	29.7 ±4.5	1.00	95.0 ±11.3	1.00	121.3 ±5.8	1.00	11.0 ±4.6	1.00	11.3 ±4.9	1.00	11.3 ±3.8	1.00	8.7 ±4.5	1.00	23.3 ±2.3	1.00	24.7 ±7.1	1.00
Agmatine Sulfate (G-Agmatine®) μg/plate																				
16	18.3 ±3.1	1.49	20.3 ±7.2	0.69	96.7 ±6.5	1.02	114.0 ±8.7	0.94	11.3 ±6.4	1.03	15.7 ±5.0	**1.38**	10.0 ±4.6	0.88	7.3 ±4.7	0.85	22.7 ±1.5	0.97	35.3 ±7.5	1.43
50	17.7 ±9.9	1.43	25.0 ±2.0	0.84	97.3 ±4.5	1.02	101.0 ±20.4	0.83	13.7 ±4.5	1.24	11.7 ±3.1	1.03	9.7 ±3.8	0.85	7.0 ±2.7	0.81	16.7 ±2.9	0.71	34.3 ±1.2	1.39
160	16.7 ±3.8	1.35	30.3 ±8.7	1.02	90.7 ±13.5	0.95	100.3 ±23.0	0.83	12.7 ±6.7	1.15	12.3 ±1.5	1.09	12.3 ±4.7	1.09	9.3 ±1.5	1.08	23.0 ±7.0	0.99	37.3 ±7.1	1.51
500	24.0 ±5.3	1.95	23.3 ±3.8	0.79	73.0 ±9.2	0.77	102.7 ±16.0	0.85	9.7 ±0.6	0.88	13.3 ±5.9	1.18	12.3 ±4.7	1.09	10.0 ±2.7	1.15	17.7 ±4.2	0.76	34.7 ±7.2	1.41
1600	18.3 ±3.8	1.49	21.3 ±7.8	0.72	78.0 ±12.1	0.82	98.7 ±22.0	0.81	11.0 ±3.0	1.00	13.0 ±2.7	1.15	10.7 ±4.9	0.94	8.0 ±3.0	0.92	27.7 ±3.5	1.19	44.0 ±5.2	1.78
5000	17.7 ±2.9	1.43	24.3 ±2.5	0.82	87.3 ±8.1	0.92	105.7 ±11.2	0.87	10.3 ±1.2	0.94	13.3 ±7.2	1.18	8.7 ±0.6	0.76	11.3 ±2.1	1.31	21.3 ±2.3	0.91	44.0 ±6.9	1.78
Positive Control																				
NPD (4 μg)	270.3[a] ±29.2	15.60	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SAZ (2 μg)	–	–	–	–	926.7[a] ±130.1	9.75	–	–	1397.3[a] ±60.6	127.03	–	–	–	–	–	–	–	–	–	–
9AA (50 μg)	–	–	–	–	–	–	–	–	–	–	–	–	474.7[a] ±156.5	54.77	–	–	–	–	–	–
MMS (2 μL)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	900.0[a] ±72.1	38.57	–	–
2AA (2 μg)	–	–	1058.7[a] ±16.7	46.71	–	–	976.0[a] ±152.6	9.07	–	–	122.3[a] ±2.5	12.23	–	–	87.3[a] ±13.5	11.39	–	–	–	–
2AA (50 μg)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	148.0[a] ±13.0	7.05

†Bacteria test strains grown in the absence (-) or presence (+) of S9 activation system, were exposed to escalating agmatine sulfate (as G-Agmatine®) concentrations along with appropriate solvent (negative) controls and positive controls and incubated at 37^0 C for 48 hr. Ultrapure water was the solvent of G-Agmatine® and of the positive control substances: SAZ (sodium azide) and MMS (Methyl methanesulfonate). DMSO (Dimethyl sulfoxide) was the solvent of the positive control substances: NPD (4-Nitro-1,2-phenylenediamine), 9AA (9-Aminoacridine) and 2AA (2-aminoanthracene). Mutation rate (MR) values of colonies treated with G-Agmatine®, SAZ, MMS and of untreated control were calculated in comparison to ultrapure water solvent values. MR values of NPD, 9AA and 2AA were calculated in comparison to DMSO solvent values. Results are expressed as mean revertants (Revs) per plate ±SD values, and as MR values, of three experiments. [a]$p < 0.05$ as compared to the relevant negative controls.

Table 4
Experiment A - Effects of 3-hour treatment with agmatine sulfate (as G-Agmatine®) on chromosome aberrations at 20 hours post-treatment (3 h Treatment/20 h Sampling) in cultured Chinese hamster lung (male) cells (V79 cell line).[†].

Group	Mean Aberrant Cells per 150 Metaphase Cells (3 h Treatment/20 h Sampling)			
	-S9		+S9	
	Including gaps	excluding gaps	Including gaps	excluding gaps
Negative control - Solvent	7 ± 0.7	4 ± 0.7	6 ± 1.4	3 ± 0
Positive Control - EMS	43 ± 2.1[a]	37 ± 2.1[a]	–	–
Positive Control - CPP	–	–	48 ± 0.7[a]	42 ± 2.1[a]
Agmatine Sulfate (G-Agmatine®) µg/ml				
500	7 ± 0	3 ± 0	7 ± 1.4	3 ± 0.7
1000	8 ± 2.1	4 ± 0.7	6 ± 1.4	3 ± 0.7
2000	7 ± 0.7	3 ± 0	9 ± 0.7	4 ± 0.7

†Cells cultured in the absence (-) or presence (+) of S9 metabolic activation system, were exposed for 3 hours (treatment) to escalating concentrations of agmatine sulfate (G-Agmatine®) and harvested at 20 hours after treatment initiation (sampling). For negative controls, cells were exposed to the solvent DME (Dulbecco's Modified Eagle's). For positive controls, cells were exposed to either Ethyl Methanesulphonate (EMS, 1.0 l/ml) or Cyclophosphamide (CPP, 5.0 g/ml). Results are the mean values of aberrant cells per 150 metaphase cells ±SD of three experiments ran in duplicates. [a]$p < 0.01$ as compared to the concurrent negative control and to historical controls.

3. Results

3.1. Ames test

No precipitation of the test item, G-Agmatine®, was observed on the culture plates in any of the examined bacterial strains grown either in the absence or presence of S9 activation system at any G-Agmatine® concentration throughout the study. The revertant colony numbers of solvent (negative) controls grown without and with S9 activation system, demonstrated similar mean numbers of spontaneous revertants characteristic of corresponding historical control data range (results not shown). In both phases of the test, the Initial Mutation Test (Table 2) and the Confirmatory Mutation Test (Table 3), the reference mutagen treatments (positive controls) induced the expected increases in revertant colonies in all the tested strains. G-Agmatine® however, did not show any significant inhibitory or cytotoxic effects. Neither the bacterial colonies nor the background lawn development were affected. Any reduced revertant colony numbers (compared to revertant colony numbers of the negative controls) remained within biological variability range of the applied test system. No biologically relevant increase in revertant colony numbers was observed in any of the five test strains following treatment with any concentration of G-Agmatine®, either in the presence or absence of S9 activation system (Tables 2 and 3).

3.2. In Vitro Mammalian Chromosomal Aberration Test

No precipitation of G-Agmatine®, and no biologically relevant changes in pH, or in osmolality of the test system (cell cultures) were observed at any of the tested G-Agmatine® concentrations (results not shown).

The number of chromosome aberrations found in cultured Chinese hamster lung (male) cells (V79 cell line), cultivated in the presence of solvent controls was compatible with the historical laboratory control data (results not shown). The concurrent positive controls ethyl methanesulphonate (0.4 and 1.0 ul/ml) and Cyclophosphamide (5 ug/ml) caused the expected biologically relevant increases of cells with structural chromosome aberrations as compared to solvent controls (Tables 4 and 5) and were compatible with historical positive control data. Thus, the study was considered valid.

In Experiment A, no biologically significant increases were observed in the number of cells with structural chromosome aberrations, cultured with or without S9 activation system, in the presence of any G-Agmatine® tested concentrations (Table 4). There were no statistical differences between G-Agmatine® treatment and concurrent solvent or historical control groups, and no dose-response relationships were observed (Table 4).

In Experiment B, the frequency of cells with structural chromosome aberrations did not show significant changes as compared to concurrent controls when cultured in the presence of escalating G-Agmatine® concentrations without S9 system over a prolonged treatment period of 20 hours and harvested at 20-, or 28-hours following treatment initiation (Table 5). Further, a 3-hour treatment interval with up to the maximum G-Agmatine® concentration (2000 µg/ml) in the presence of S9 system did not cause an increase in the number of cells with structural chromosome aberrations when harvested 28 hours following treatment initiation (Table 5).

No polyploid cells or end reduplicated cells were observed in any of the experiments (results not shown).

Table 5
Experiment B - Effects of agmatine sulfate (as G-Agmatine®) treatment for 20 hours (Experiment B.I and II) or for 3 hours (Experiment B.III) on chromosome aberrations in cultured Chinese hamster (male) lung cells (V79 cell line) when harvested at 20 hours (Experiment B.I) or 28 hours (Experiment B.II and III) post-treatment initiation (sampling) intervals.[†].

Group	Mean Aberrant Cells per 150 Metaphase Cells						
	Experiment B.I -S9 20 h Treatment/20 h Sampling		Experiment B.II -S9 20 h Treatment/28 h Sampling		Group	Experiment B.III +S9 3 h Treatment/28 h Sampling	
	Including Gaps	Excluding Gaps	Including Gaps	Excluding Gaps		Including Gaps	Excluding Gaps
Negative Control Solvent	6± 1.4	3± 0.7	7 ± 0	4 ± 0	Negative control Solvent	7 ± 0.7	3 ± 0
Positive Control EMS	44 ± 2.1[a]	38 ± 4.2[a]	42 ± 0.7[a]	36 ± 4.2[a]	Positive Control CPP	45 ± 1.4[a]	41 ± 2.1[a]
Agmatine Sulfate (G-Agmatine®) µg/ml					Agmatine Sulfate (G-Agmatine®) µg/ml		
375	6 ± 0	3 ± 0.7	7 ± 0.7	3 ± 0	500	7 ± 1.4	3 ± 0
750	7 ± 0.7	3 ± 0	7 ± 0	4 ± 0	1000	8 ± 0.7	4 ± 0
1500	8 ± 0	4 ± 0	7 ± 0	3 ± 0	2000	7 ± 0.7	4 ± 0

†Cells cultured in the absence (-) or presence (+) of S9 activation system, were exposed for varied times to escalating concentrations of agmatine sulfate (as G-Agmatine®) and harvested at various intervals thereafter. For negative controls, cells were exposed to the solvent DME (Dulbecco's Modified Eagle's). For positive controls, cells were exposed to either Ethyl Methanesulphonate (EMS, 4.0 l/ml) or Cyclophosphamide (CPP, 5.0 g/ml). Results are the mean values of aberrant cells per 150 metaphase cells ±SD of three experiments ran in duplicates. [a]$p < 0.01$ as compared to the concurrent negative control and to historical controls.

Table 6
Effect of oral agmatine sulfate (as G-Agmatine®) treatment on the numbers and frequencies of micronucleated polychromatic erythrocytes after 24- or 48-hour intervals.[†].

Groups (n)	Male		Female	
	MPCE	PCE/ NCE	MPCE	PCE/ NCE
Sample Time – 24 hours				
Negative Control – Solvent (5)	5.20 0.84	1.22 0.07	5.20 0.84	1.25 0.14
Positive Control –CPP 60 mg/kg (5)	126.40 5.59[a]	0.61 0.10[a]	122.60 10.83[a]	0.55 0.20[a]
Agmatine Sulfate (G-Agmatine®) mg/kg				
500 (5)	4.20 1.10	1.20 0.05	5.00 0.71	1.20 0.08
1000 (5)	5.40 1.14	1.15 0.19	5.40 1.14	1.16 0.15
2000 (5)	5.80 0.84	1.04 0.08	5.60 0.55	1.11 0.13
Sample Time – 48 hours				
Negative Control – Solvent (10)	5.20 0.84	1.21 0.16	5.40 0.55	1.24 0.22
G-Agmatine® – 2000 mg/ kg (10)	5.60 1.52	1.08 0.07	6.00 1.00	1.07 0.07

†Oral administration volumes of agmatine sulfate (as G-Agmatine®) and solvent (aqua ad injectabilia) were 10 ml/kg. Cyclophosphamide (CPP) was administered by intraperitoneal (ip) injection in a volume of 10 ml/kg. NCE, normochromatic erythrocyte; MPCE, number of micronucleated polychromatic erythrocyte per 4000 PCE; PCE, polychromatic erythrocytes. The total number of PCE analysed in each group was 20,000. Results are the mean SD values. [a]$p < 0.01$ as compared to negative controls.

3.3. *In Vivo Mouse Micronucleus Assay*

Single oral administrations of 500, 1000, or 2000 mg/kg agmatine sulfate (as G-Agmatine®) did not lead to an increase in the frequency of MPCE in male or in female mice at either 24-, or 48-hour intervals after the treatment, as compared to concurrent control groups (Table 6).

The frequency of micronucleated polychromatic erythrocytes (MPCE) in the negative control groups were compatible with the historical control data for this laboratory (results not shown).

Cyclophosphamide treated mice (60 mg/kg, ip) showed a large, statistically significant increase in the MPCE number compared to the negative and historical controls, demonstrating the validity of the test (Table 6).

4. Discussion

The present study used currently accepted mutagenicity and genotoxicity tests as recommended by the Organization for Economic Cooperation and Development (OECD) for safety evaluation of dietary ingredients and supplements [21–23]. Using this battery of assays, namely: bacterial gene mutation test (i.e., Ames test), *in vitro* mammalian chromosomal aberration assay, and *in vivo* rodent hematopoietic cells chromosomal damage test (i.e., mouse micronucleus test), results of the present study provide further evidence for the safety of G-Agmatine®, a proprietary brand of agmatine sulfate.

Results of the Ames test mutagenicity assay show that under the experimental conditions applied in the present study, G-Agmatine® did not induce gene mutations as adjudged by base pair changes or frameshifts in the genome of the strains used. No significant increases were observed in revertant colony numbers of any of the five test strains following treatment with G-Agmatine® at any of the studied concentrations, either in the presence or absence of metabolic activation system (S9). Sporadic observed increases in revertant colony numbers were considered biologically irrelevant, reflecting the variability of the test system. No tendency for higher mutation rates was observed with increasing G-Agmatine® concentrations. The biological relevance of the results is considered the main criterion for result interpretations. It is pertinent to note that negative Ames test is also considered a good predictor of non-carcinogenicity [37].

When assayed for genotoxicity in the chromosomal aberration test, a test item is considered clearly negative if: 1) none of the tested concentrations exhibit a statistically significant increase in structural chromosome aberrations as compared to concurrent negative control; 2) there is no concentration-related increase when evaluated against an appropriate trend test, and 3) all results are inside the distribution of the laboratory historical negative control data. When tested up to the maximum concentration of 2000 μg/ml, both with and without S9 metabolic activation system (three-hour treatment) and without S9 system (twenty-hour treatment), G-Agmatine® did not induce structural chromosome aberrations in Chinese Hamster lung cells. No statistically significant differences between G-Agmatine® treatment and concurrent solvent control groups, and no dose-response relationships were observed. All the observed chromosomal aberrations were inside the distribution of the laboratory historical negative control data. Additionally, there were no biologically relevant increases in the rate of polyploid or endo-reduplicated metaphases in either experimental exposures both in the presence or absence of S9 metabolic activation system.

Using the *in vivo* micronucleus assay, a test item is considered to lack genotoxic activity if: 1) an increase in the frequency of micronucleated polychromatic immature erythrocytes (MPCE) is not observed in the treatment groups when compared to the concurrent negative control; 2) there is no dose-related increase at any sampling time, and 3) all results are inside the distribution of historical negative control data. Results of the present study show no biological or statistically significant increases in the frequency of MPCE in the groups of mice treated with G-Agmatine® as compared to the vehicle control group.

In conclusion, the present study shows for the first time that agmatine sulfate has no mutagenic effect in the bacterial Ames test. It is non-clastogenic as shown by the *in vitro* chromosomal aberration test using Chinese Hamster lung cells. And it lacks genotoxic effects following oral administration as evidenced by the mouse micronucleus test. Taken together with previous findings from laboratory animals [4,7], companion animals [32,33], and human studies [8,17,27], results of the present study provide further support for the safety of agmatine sulfate as a dietary ingredient.

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CRediT authorship contribution statement

Gad M. Gilad: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Erzsébet Béres:** Writing – original draft, Validation, Resources, Methodology, Investigation, Funding acquisition. **Varda H. Gilad:** Resources, Project administration, Funding acquisition, Conceptualization. **Gábor Hirka:** Writing – original draft, Validation, Resources, Methodology, Investigation, Formal analysis. **Adél Vértesi:** Writing – original draft, Validation, Resources, Methodology, Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. GAD M. GILAD, PhD reports was provided by Gilad&Gilad LLC. GAD M. GILAD, PhD reports a relationship with Gilad&Gilad LLC that includes: employment and equity or stocks. GAD M. GILAD, PhD has patent licensed to Gad M. Gilad. Gad M. Gilad reports a relationship with Gilad&Gilad LLC that includes: employment, equity or stocks, and

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Data availability

Data will be made available on request.

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