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Evidence for oral agmatine sulfate safety – A 95-day high dosage pilot study with rats

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ABSTRACT

Agmatine, decarboxylated arginine, exerts beneficial effects in various experimental disease models. Clinical trials indicate the safety and effectiveness of short-term (up to 21 days) high dose regimens of oral agmatine sulfate, but longer term studies are lacking. This pilot study undertook to assess the safety of a longer term high dosage oral agmatine sulfate in laboratory rats. Adult Wistar rats consumed 5.3 g/l agmatine sulfate in their drinking water for 95 days, a regimen estimated to result in a daily dosage of absorbed agmatine of about 100 mg/kg. Animals' body weight, water consumption and blood pressure were periodically measured, and general cage behavior, fur appearance, urination and feces appearance monitored. These parameters were also determined at 20 days after treatment cessation (day 115). On days 95 and 115, animals were euthanized for gross necropsy assessment. Agmatine-treated rats showed slight, but significant reductions in body weight and blood pressure, and reduced water consumption during treatment, which recovered completely within 20 days after treatment cessation. Otherwise, no abnormal behaviors or organ pathologies were observed. These findings are first to suggest apparent safety of sub-chronic high dosage dietary agmatine sulfate in laboratory rats, thus lending further support to the therapeutic applications of agmatine.

1. Introduction

Agmatine, also known as decarboxylated arginine, is a naturally occurring molecule ubiquitously found in living organisms (Kossel, 1910; Tabor and Tabor, 1984). Substantial pre-clinical body of evidence recently reviewed by Piletz et al. (2013), shows that agmatine treatment exerts beneficial cytoprotective effects on various bodily functions (e.g., neuroprotection, nephroprotection, cardioprotection and gastroprotection) and in various disease models including: diabetes, neurotrauma (e.g., stroke, brain and spinal cord injury, glaucoma), neuropathic pain, opioid analgesia and addiction, neurodegenerative diseases (e.g., Parkinson's disease) and psychiatric diseases (e.g., anxiety, depression).

Evidently, agmatine interacts with and modulates multiple molecular targets and thereby exerts its beneficial effects. These targets were recently summarized by Piletz et al. (2013) and include: (A) modulation of several neurotransmitter receptors and receptor ionophores; (B) blockade of key ionic channels; (C) inhibition of membrane transporters; (D) modulation of nitric oxide (NO) formation; (E) modulation of polyamine metabolism; (F) inhibition of protein ADP-ribosylation and thus, interfering with cell signaling; (G) inhibition of matrix metalloproteases (MMPs), enzymes implicated in nerve cell death and neuropathic pain; and (H) inhibition of advanced glycation end (AGE)-product formation, a process involved in the pathology of diabetes and neurodegenerative diseases.

While agmatine is produced endogenously by arginine decarboxylation in mammals (Li et al., 1994), it is also acquired from the diet where, as summarized by Molderings et al. (2003), it is found in low amounts in a wide variety of foodstuff from plants, fish and animals. Additionally, many gastrointestinal (GI) bacteria synthesize agmatine as predominant precursor for polyamine biosynthesis (Burrell et al., 2010), and some bacteria produce and secrete agmatine as a mechanism to resist the extreme acidity of the stomach (Tsai and Miller, 2013). Therefore, the significant concentrations of agmatine found in the mammalian GI tract implicate microbial production as the main source of systemic agmatine (Haenisch et al., 2008; Molderings et al., 2003; Swanson et al., 2002). Animal studies demonstrated that exogenous agmatine sulfate, the commonly used salt form of agmatine, is absorbed in the GI tract and then rapidly (within minutes) distributed throughout

Abbreviations: AGE, advanced glycation end product; GABA, γ-aminobutyric acid; GI, gastrointestinal; MMPs, matrix metalloproteases; NO, nitric oxide; SBP, systolic blood pressure.

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the body (Molderings et al., 2003), including the brain (Piletz et al., 2003). In humans, ingested agmatine is readily absorbed and elimination of unmetabolized agmatine by the kidneys indicates an apparent blood half life of about 2 h (Huisman et al., 2010).

In the body, agmatine is principally metabolized into urea and putrescine the diamine precursor of polyamines, which are not only essential for cell proliferation (Tabor and Tabor, 1984), but also for viability of mature cells in general and specifically so for nerve cells (Gilad et al., 1996a,b). Agmatine-derived putrescine may also serve as a minor precursor for the neurotransmitter GABA (γ-aminobutyric acid) (Molderings et al., 2003). Additionally, agmatine can also be oxidized resulting in agmatine–aldehyde formation, which may be toxic and secreted by the kidneys (Lortie et al., 1996; Satriano et al., 2001). This latter route is tissue specific, being significant in some tissues (Cabella et al., 2001; Molderings et al., 2003), but minor in others (Bhagvat et al., 1939; Taylor and Leiber, 1979) and apparently negligible in the central nervous system (Piletz et al., 2013). In the kidneys, agmatine can moderately increase glomerular filtration rate and cause natriuresis (Lortie et al., 1996), an effect which may be desired or unwanted depending on health status. Additionally, agmatine has been implicated in increase gastric acid secretion, which in turn may increase incidence of stress-induced ulcers in laboratory rats (Glavin et al., 1995; Molderings et al., 2003).

Taken together, the evidence indicates that agmatine is a potential therapeutic for several disorders, but its interaction with multiple targets raises concerns for induction of unwanted side effects, especially with longer term treatment. Almost all animal studies thus far reported the effects of short-term (usually up to 7 days) systemic agmatine treatments using a daily dose range of 50–100 mg/kg (Gilad et al., 1996c; Piletz et al., 2013), but one study recently reported a longer term, 4 to 6 weeks, treatment with a daily intraperitoneal dose of 40 mg/kg (Rushaidhi et al., 2013). Few studies using oral agmatine administration in infant mice (Moore et al., 2001), adult rats (Molderings et al., 2003) and calves (Harp and Waters, 2000) claimed no adverse effects by agmatine itself at doses ranging between 64 and 500 mg/kg, but these studies also were of short (up to 8 days) durations. Importantly, recent human clinical trials found high doses of oral agmatine sulfate (1335–3560 mg/day) to be safe and effective when taken for periods of up to 21 days, with low incidence of mild diarrhea reported at the highest used dosage (Keynan et al., 2010). In view of this evidence and the intense interest in agmatine's therapeutic potential (Molderings and Haenisch, 2012; Piletz et al., 2013; Uzbay, 2012), assessment of the longer term effects of oral agmatine treatment is clearly required. We therefore, undertook a pilot study to assess whether a longer term (95 days) high dosage oral agmatine sulfate regimen has any general toxic effects in laboratory rats.

Of specific concern, in view of previous reports on stress-induced stomach ulcers (Glavin et al., 1995; Molderings et al., 2003), it was important to examine whether or not such agmatine regimen has any ulcerogenic effects. For this reason, agmatine was administered via the drinking water, rather than by the stressful gavage (or forced-) feeding procedure, to avoid treatment-associated chronic intermittent stress. Additionally of interest, was to determine whether oral agmatine may produce similar mild blood pressure reduction as do systemic agmatine administrations (Raasch et al., 2001).

2. Materials and methods

2.1. Animals

Experiments were performed at the Laboratory of Neuroscience in Assaf Harofeh Medical Center, with 75- to 80-day-old female ($n = 20$) and male ($n = 20$) Wistar rats (Harlan, Israel). After arrival, the animals were acclimatized for 5 days prior to treatment. The animals were kept in individual cages under standard conditions of temperature (22 ± 2 °C), humidity (55–75%), and light (12 h light–dark cycle, lights on at 06:00 AM) with a free supply of food (pellet rat chow) and tap water in an accredited vivarium (Israel Ministry of Health) according to the Institutional Animal Care and Use Committee (Assaf Harofeh Medical Center) approved protocols.

2.2. Treatments

Female and male animals were separately assigned randomly into experimental ($n = 10$) and control ($n = 10$) groups and experiments began by giving the experimental animals drinking water containing 5.3 g/l of agmatine sulfate, $H_2N(CH_2)_4$-$NHC(=NH)NH_2 \cdot H_2SO_4$ (CAS No. 2482-00-0), synthetically manufactured as a dietary ingredient according to international standards (ISO 9001).

2.2.1. Dosage justification

The rationale for choosing the present dosage was to achieve high systemic agmatine sulfate concentrations, equivalent to the highest systemic dose used in rat studies, while at the same time to avoid any unwanted GI effects which might arise with longer term oral administration of such a high dose. The GI absorption *efficiency* of agmatine sulfate over time has not been accurately determined previously. Predicting drug absorption is difficult and the overall estimate of bioavailability is complex (Burton et al., 2002). Therefore, based on absorption principles assuming Class 3: high solubility-low permeability absorption for agmatine (Amidon et al., 1995) and assuming membrane permeation by selective transporters subject to competition with other substrates [e.g., polyamines (Molderings et al., 2003)] operating in the same or opposite directions (Pang, 2003) and cellular enzymatic activities that may reduce its concentration (Pang, 2003), we have arbitrarily estimated a conservative low value of 10% for agmatine sulfate GI absorption. Previously collected records at our vivarium using groups of female ($n = 10$) and male ($n = 15$) rats matched to the present study design, indicated that rats with an average body weight of 204.0 ± 1.4 g drank an average volume of 38.5 ml/day. Accordingly, a concentration of 5.3 g/l agmatine sulfate would result in consumption of 204.05 mg/day or, based on a 204 g body weight, in a daily dosage of 1000 mg/kg. At 10% absorption, this dosage is estimated to lead to 20.405 mg per rat, which would translate to a systemic daily dose of 100.02 mg/kg, a value equivalent to the highest systemic doses used in rodent studies (Gilad et al., 1996c).

Freshly prepared water supply was given every 2 days. Agmatine sulfate water solution is stable at room temperature for at least 3 days (measured by mass spectrometry; results not shown) and has a slightly bitter taste. Control animals were given regular tap drinking water. Rats were maintained on this diet for 95 days. A group of experimental male rats ($n = 5$) were then switched to regular drinking water (without agmatine sulfate) for additional 20 days (day 115).

2.3. Measures

The following measurements were performed at day 1 (the day immediately prior to starting date of the experiment) and at various intervals thereafter: total body weight, drinking water volume and blood pressure. Animals were also observed twice weekly in their home cage prior to cage cleaning for changes in fur appearance (cleanliness or hair loss) and feces appearance. Urine volumes were not measured, but cages were inspected for any gross changes in urination. General cage behavior was not quantified, but examined for 5 min beginning 30 min after cage cleaning, between 10:00 and 12:00 AM, for any gross changes in locomotion (moving around the cage), general activity (upright standing, sniffing and digging) and self-grooming (body fur and paw licking) (Saibaba et al., 1996).

For blood pressure measurements, rats were acclimated to a movement-limiting Plexiglas chamber for 15–20 min, tail vasodilatation was produced with a heat lamp and Systolic blood pressure was measured by tail-cuff plethysmography (IITC Life Science, Woodland Hills, CA) and the average of five replicate measurements was recorded.

For gross necropsy, groups of male rats were deeply anesthetized with an overdose of sodium pentobarbital (750 mg/kg intraperitoneally) and euthanized on day 95 of treatment and at 20 days after treatment cessation (day 115), and the stomach, liver, kidney, heart, spleen and brain were excised for macroscopical examination and weighed. For ulcer examination, the stomach was opened along the greater curvature, the lumen was rinsed with saline, and the lining mucosa was inspected macroscopically under transmitted light for any appearance of ulcer lesions (Yelken et al., 1999).

2.4. Statistical analysis

Results were tabulated and presented by descriptive statistics as means and standard error of the means (SEM). Statistical analysis was performed using SPSS (Statistical Package for Social Sciences, v. 15.0, SPSS Inc., Chicago, IL). For continuous data analyses, we used repeated measures ANOVA and 2-tailed paired *t*-test. To better appreciate changes, data were normalized by plotting changes as the percent of pretreatment (day 1) values. Differences were considered significant at $p \leqslant 0.05$.

3. Results and discussion

3.1. Changes in water intake and body weight

As summarized in Table 1, agmatine sulfate-drinking animals showed reduction in water intake volume and a concomitant slight weight loss during the first 21 days of treatment which was thereafter, followed by renewed weight gain. The reduction in water intake persisted for the duration of agmatine sulfate treatment. Animals switched to regular drinking water after 95 days demonstrated a complete recovery in drinking water consumption within 20 days and showed ongoing weight gain (Table 1). The significance of these differences is better appreciated when changes are graphically illustrated as percent of day 1 baseline values (Fig. 1).

The post-treatment recovery may indicate that the slight bitter taste of agmatine sulfate-containing water might have been the cause of the reduction in water intake, which in turn affected body weight gain. In this regard, it is worth noting that systemic (intraperitoneal) agmatine sulfate injection may rather *increase* caloric intake with carbohydrate preference in satiated, but not hungry rats (Prasad and Prasad, 1996). And this effect may be mediated by neuropeptide Y in the hypothalamus paraventricular and arcuate nuclei (Taksande et al., 2011). In human clinical trials, weight gain changes were not observed in individuals taking high daily dose of oral agmatine sulfate for up to 21 days (Keynan et al., 2010). Furthermore, there are no reports of weight gain changes by individuals who are currently taking high daily agmatine sulfate doses (1.78–2.67 g/day) as a dietary ingredient for periods exceeding 1 year (Gilad GM and Gilad VH, unpublished observations).

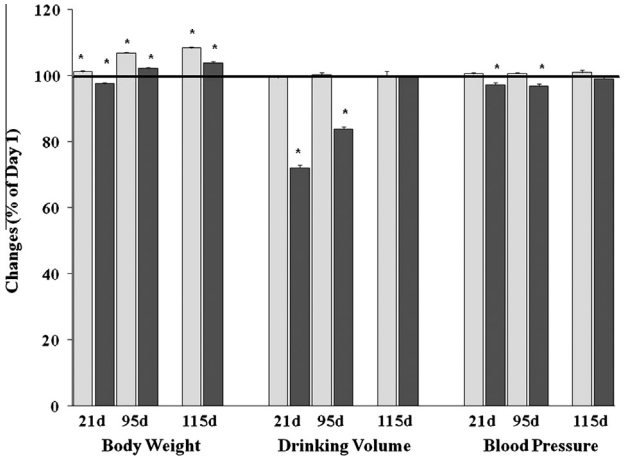

Fig. 1. Changes in body weight, daily drinking volume and systolic blood pressure of rats during (21 and 95 days), and at 20 days (day 115) after cessation of drinking agmatine sulfate containing water (dark bars) as compared to controls (light bars). Results, mean ± SEM values of 10 animals (or 5 animals in the 115-day group), are expressed as percent of baseline (day 1) values (black horizontal line). * = $p \leqslant 0.05$; asterisks above the light bars indicate significant differences as compared to day 1; asterisks above dark bars indicate significant differences between agmatine sulfate and control groups.

3.2. Effective dosage

The actual daily agmatine sulfate consumption can be calculated based on the average values of water drank per day by females and male rats: 32.10 ml and 27.15 ml, respectively [average of female and male group values at day 21 (30.7 and 25.1 ml, respectively) and day 95 (33.5 and 29.2 ml, respectively) of treatment (Table 1)]. Given these values and agmatine sulfate water concentration of 5.3 mg/ml, then the average daily agmatine intake was 170.13 mg for females and 143.89 mg for males. Or when calculated per kg body weight [using average body weights at days 21 and 95 of treatment: 205.00 g for female and 253.10 g for male rats (Table 1)], female rats consumed an estimated 829.85 mg/kg and males 568.51 mg/kg agmatine sulfate per day. Based on an estimated 10% GI absorption rate (see Section 2.2.1), these ingested daily amounts are considered sufficient to provide agmatine levels in the circulation approaching the highest commonly used dosages, 50–100 mg/kg by systemic administrations (Gilad et al., 1996c).

When extrapolating dose range from rodent studies, an arbitrary one order of magnitude reduction is commonly used for human clinical studies (Center for Drug Evaluation and Research, 2002; Reagan-Shaw et al., 2008). Indeed, calculations similar to those described here have been used in planning the dose range for the first reported clinical trials with agmatine sulfate where safe and effective blood concentrations in humans were estimated

Table 1
Changes in female (A) and male (B) body weight, daily drinking volume and systolic blood pressure (BP) at baseline (pretreatment day 1), during (days 21 and 95) and in males at 20 days (day 115) after cessation of drinking agmatine sulfate containing water, as compared to controls.

Body weight (g) Experimental day			Daily drinking (ml) Experimental day			BP (mm Hg) Experimental day		
1	21	95	1	21	95	1	21	95
(A)								
Control								
201.8 ± 1.1	207.8[b] ± 1.1	219.0[c] ± 1.5	37.4 ± 0.2	38.1 ± 0.2	38.4[a] ± 0.1	108.3 ± 0.4	108.0 ± 0.4	108.3 ± 0.5
Agmatine sulfate								
206.8 ± 0.7	*201.5*[b] ± 0.7	208.5 ± 0.5	38.4 ± 0.2	*30.7*[c] ± 0.4	*33.5*[c] ± 0.3	109.8 ± 0.4	*106.5*[b] ± 0.5	*106.5*[b] ± 0.5

Body weight (g) Experimental day				Daily drinking (ml) Experimental day				BP (mm Hg) Experimental day			
1	21	95	115	1	21	95	115	1	21	95	115
(B)											
Control											
249.2 ± 0.8	252.2[a] ± 0.8	266.4[c] ± 0.7	274.4[c] ± 1.2	34.8 ± 0.1	34.6 ± 0.2	34.9 ± 0.2	35.0 ± 0.6	103.7 ± 0.4	104.3 ± 0.4	104.4 ± 0.3	104.2 ± 0.9
Agmatine sulfate											
253.4 ± 0.7	*247.3*[b] ± 1.0	258.9[a] ± 0.7	261.4[b] ± 1.1	34.8 ± 0.1	*25.1*[c] ± 0.2	*29.2*[c] ± 0.2	34.9 ± 0.1	106.4 ± 0.6	*103.5*[b] ± 0.7	*103.1*[b] ± 0.6	105.8 ± 0.5

Results are the mean ± SEM values of 10 animals (or 5 animals in day 115 group). Significantly *decreased* values are italisized.
[a] Differences are significant as compared to day 1 at $p < 0.05$.
[b] Differences are significant as compared to day 1 at $p < 0.01$.
[c] Differences are significant as compared to day 1 at $p < 0.001$.

Table 2
Organ weights (gram wet weight) of female (A) and male (B) rats on the 95th day of drinking agmatine sulfate-containing water (day 95) and of males (B) at 20 days (day 115) after cessation of treatment as compared to controls.

Group	Stomach	Liver	Kidney	Heart	Spleen	Brain
(A)						
Day 95						
Control	1.84 ± 0.18	8.70 ± 0.70	1.77 ± 0.12	0.95 ± 0.07	0.55 ± 0.06	1.75 ± 0.10
Agmatine Sulfate	1.95 ± 0.25	9.04 ± 0.95	1.87 ± 0.20	0.90 ± 0.08	0.70 ± 0.09	1.80 ± 0.15
(B)						
Day 95						
Control	2.04 ± 0.23	11.20 ± 0.95	2.05 ± 0.18	1.06 ± 0.09	0.65 ± 0.06	1.89 ± 0.11
Agmatine Sulfate	2.29 ± 0.31	12.34 ± 1.15	2.15 ± 0.20	0.99 ± 0.08	0.80 ± 0.09	1.92 ± 0.17
Day 115						
Control	2.24 ± 0.28	11.87 ± 0.97	2.00 ± 0.17	1.03 ± 0.06	0.62 ± 0.05	1.93 ± 0.15
Agmatine Sulfate	2.11 ± 0.24	11.56 ± 1.27	2.10 ± 0.18	1.07 ± 0.09	0.75 ± 0.08	1.97 ± 0.15

Results are the mean ± SEM values of 5 animals.

at between 2.5 and 10 mg/kg of body weight. A value which, based on an estimated GI absorption efficiency of 10%, meant an oral dose of between 1250 and 7500 mg/day for a 50–75 kg body weight and, after practical manufacturing considerations, was translated to a daily dose range of 1335–3560 mg/day (Keynan et al., 2010). Interestingly, this calculated agmatine dosage is within similar range as the recommended arginine daily dose range of 500–6000 mg/day (Wu et al., 2000).

3.3. Changes in blood pressure

Blood pressure showed a slight, but significant reduction in agmatine sulfate-treated rats (Table 1, Fig. 1). This may confirm the long known effects of high systemic agmatine administrations (up to 100 mg/kg) on lowering blood pressure and heart rate in rat studies (Raasch et al., 2001). Alternatively, the lower water intake may have resulted in reduced blood volume, which in turn caused blood pressure reduction. The reduction in blood pressure also showed complete recovery when examined at 20 days after treatment cessation (Table 1, Fig. 1). It is important to note that no significant cardiovascular changes were observed in human clinical trials with participants taking high dose oral agmatine sulfate for up to 21 days (Keynan et al., 2010).

3.4. General cage behavior

No apparent differences in general cage behavior, fur appearance, feces appearance, or urination were observed over time between agmatine-treated and control groups (results not shown).

3.5. Gross necropsy

No apparent changes were observed in gross necropsy and neither in organ weights (Table 2). Importantly, no stomach ulcer lesions were observed in any of the groups. While prior studies have implicated agmatine sulfate in aggravating stomach ulcers in stressed rats (Glavin et al., 1995; Molderings et al., 2003), the present study indicates that agmatine is not ulcerogenic when administered orally to normal healthy rats. It should be noted that the highest endogenous agmatine concentrations were found in rat stomach (Raasch et al., 1995; Stickle et al., 1996) and new evidence rather points out its potential role in cytoprotection against gastric acid damage (Steer, 2009). Furthermore, clonidine has been shown to inhibit stress induced ulcers in rats (Yelken et al., 1999) and agmatine, as an alpa 2-adrenoceptor ligand and a clonidine-displacing substance (Pinthong et al., 1995) might have been expected to do the same. Moreover, a recent study shows that agmatine sulfate pretreatment (intraperitoneally, 100 mg/kg), immediately prior to experimental gastric ischemia in rats, exerts protection against gastric tissue damage (Al Masri and Eter, 2012).

Interestingly, *PubChem Compound* website: http://pubchem.ncbi.nlm.nih.gov (Agmatine sulfate, 2013) summarizes data from high-throughput screening assays and shows 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). This is taken as evidence that agmatine sulfate is less likely to exert non-specific toxic side effects or to be involved in interactions with other drugs.

In vitro studies indicate 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 (Whitfield et al., 1962, 1968), and, more recently, of neural stem cells (Kuo et al., 2011; Song et al., 2011), it also exerts rather anti-proliferative effects, on various other cell types including smooth muscle cells, macrophages, fibroblasts, astrocytes, as well as cancer cells (Gilad et al., 1996c; Kuo et al., 2011; Molderings et al., 2004; Regunathan et al., 1996; Song et al., 2011). Apparently, the effects of agmatine on cell proliferation depend on cell type and on the stage of cellular differentiation. Importantly, while agmatine may be considered a cytotactic agent for certain cell types, it is however, not cytotoxic (Gilad et al., 1996c; Molderings et al., 2004).

4. Conclusions

This pilot study provides for the first time initial evidence suggesting the apparent safety of sub-chronic high dosage oral agmatine sulfate treatment in laboratory rats. Although these initial positive findings are limited in scope, they now set the stage for follow up studies required to ascertain the lack of agmatine's long term toxicity including: microscopic histopathology examinations, toxicokinetics including maximum tolerated dose determinations, and blood and urine analyzes for organ biomarker characterizations. In spite of this shortcoming however, the present study is important not only because it further supports the ongoing use of agmatine as a safe dietary ingredient, but also since this evidence of safety highlights the potential of agmatine's therapeutic applications.

Conflict of Interest

Gad M. Gilad and Varda H. Gilad hold stocks in Gilad&Gilad LLC and are co-owners of intellectual property and patents related to agmatine usage.

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