Inhibition of adenosine A1 receptors abolished the nutritional ketosis-evoked delay in the onset of isoflurane-induced anesthesia in Wistar Albino Glaxo Rijswijk rats

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Research article

Keywords: isoflurane, immobility, exogenous ketone supplements, ketosis, adenosine receptors

Posted Date: October 25th, 2019

DOI: https://doi.org/10.21203/rs.2.16472/v1

Version of Record: A version of this preprint was published at BMC Anesthesiology on January 30th, 2020. See the published version at https://doi.org/10.1186/s12871-020-0943-z.

Abstract

Background It has been demonstrated that administration of exogenous ketone supplement ketone salt (KS) and ketone ester (KE) increased blood ketone level and delayed the onset of isoflurane-induced anesthesia (immobility) in different rodent models, such as Wistar Albino Glaxo Rijswijk (WAG/Rij) rats. The modulatory effect of adenosinergic system may have a role in the ketone supplementation-evoked effects on isoflurane-generated anesthesia. Thus, we investigated whether adenosine receptor antagonists can modulate the effect of exogenous ketone supplements on the onset of akinesia induced by isoflurane.

Methods To investigate the effect of exogenous ketone supplements on anesthetic induction we used ketone supplement KE, KS, KEKS (1:1 mix of KE and KS), KSMCT and KEMCT (1:1 mix of KS and KE with medium chain triglyceride/MCT oil, respectively) in WAG/Rij rats. Animals were fed with standard diet, which was supplemented by oral gavage of different ketone supplements (2.5 g/kg/day) for 1 week. After 7 days, isoflurane (3%) was administered for 5 min and the time until onset of isoflurane-induced anesthesia (immobility) was measured. Changes in levels of blood β-hydroxybutyrate (βHB), blood glucose and body weight of animals were also recorded. To investigate the putative effects of adenosine receptors on ketone supplements-evoked influence on isoflurane-induced anesthesia we used a specific adenosine A1 receptor antagonist DPCPX (intraperitoneally/i.p. 0.2 mg/kg) and a selective adenosine A2A receptor antagonist SCH 58261 (i.p. 0.5 mg/kg) in combination with KEKS.

Results Significant increases were demonstrated in both blood βHB levels and the number of seconds required before isoflurane-induced anesthesia (immobility) after the final treatment by all exogenous ketone supplements. Moreover, this effect of exogenous ketone supplements positively correlated with blood βHB levels. It was also demonstrated that DPCPX completely abolished the effect of KEKS on isoflurane-induced anesthesia (immobility), but not SCH 58261.

Conclusions These findings strengthen our previous suggestion that exogenous ketone supplements-evoked increase in ketone levels (ketosis) might affect surgical anesthetic needs. Moreover, our results demonstrate that adenosinergic system (likely through A1Rs) may modulate the influence of exogenous ketone supplements-evoked ketosis on isoflurane-generated anesthesia.

Background

It has been demonstrated that exogenous ketone (ketogenic) supplements, such as ketone ester (KE), not only increase the level of ketone bodies (e.g., β-hydroxybutyrate/βHB) [1–5], but also maintain blood levels of ketone bodies in both animals and humans [2,3,6]. Ketone bodies, such as βHB, enter into the brain through blood-brain barrier and provide fuel to brain cells [7,8] improving cell energy metabolism (e.g., enhance mitochondrial ATP synthesis) [9]. Moreover, ketone supplement-induced ketosis can suppress neuronal excitability [7,10,11], modulate functions of ion channels and neurotransmitter systems (e.g., increase GABA and adenosine levels) [7,12–14] and influence inflammatory processes

(e.g., decrease the concentration and expression of proinflammatory cytokines) [15]. It was suggested that these effects of ketosis may have therapeutic potential in the treatment of several central nervous system (CNS) diseases, such as Alzheimer's disease, Parkinson's disease, epilepsy and psychiatric disorders (e.g., anxiety, schizophrenia and depression) [1,3,8,16]. It was also demonstrated that exogenous ketone supplements, such as KE and ketone salt (KS) are relatively well-tolerated without (or with minimal) adverse effects [1,2,6,16,17]. However, exact mechanism(s) of action of exogenous ketone supplement-generated ketosis on CNS diseases and other pathophysiological and physiological processes are largely unknown.

It was suggested that ketosis may modulate sleep and sleep-like effects [18–22]. Indeed, it has been demonstrated recently that nutritional ketosis (evoked by exogenous ketone supplements, such as KE) delayed the onset of inhalational anesthetics isoflurane (1-chloro–2,2,2-trifluoroethyl difluoromethyl ether)- induced anesthesia (immobility) [23]. Nevertheless, mechanism of action of ketosis-induced changes in isoflurane-evoked anesthesia remains unknown. It was suggested that changes, for example, in functioning of different ion channels (e.g., K_{ATP} channels), neurotransmitter systems (e.g., GABAergic and adenosinergic system) and mitochondria (e.g., mitochondrial respiration) may have a role in ketone supplement-evoked effects on isoflurane-generated anesthesia [19,23–26]. However, it has also been demonstrated that ketosis (evoked by exogenous ketone supplements) [1,2,4,5] may increase adenosine level in the brain [14] and adenosine may have a role not only in the sleep [27], but also the generation of sleep-like effects [28,29]. Therefore, in this study, we examined the effect of ketone supplement KE, KS and their mix (KEKS), as well as mix of KS and KE with medium chain triglyceride (MCT) oil (KSMCT and KEMCT, respectively) on isoflurane-induced onset of anesthesia (latency to immobility). Animals (Wistar Albino Glaxo Rijswijk/WAG/Rij rats) were fed with standard diet and were gavaged with different ketogenic supplements for 1 week (2.5 g/kg/day). After the last supplement gavage we recorded the time until anesthetic induction (under 3 % isoflurane), defined as the onset of immobility. In the second part of the study, the potential role of adenosine receptors in the nutritional ketosis-evoked effects on isoflurane-induced onset of anesthesia (immobility) was investigated. We used a specific adenosine A1 receptor (A1R) antagonist DPCPX (1,3-dipropyl–8-cyclopentylxanthine) (intraperitoneally/i.p. 0.2 mg/kg) and a selective adenosine A2A receptor (A2AR) antagonist SCH 58261 (7-(2-phenylethyl)–5-amino–2-(2-furyl)-pyrazolo-[4,3-e]–1,2,4-triazolo[1,5-c]pyrimidine) (i.p. 0.5 mg/kg) in combination with KEKS (2.5 g/kg/day, gavage).

This study is the continuation of our previous study on genetically absence epileptic WAG/Rij rat strain (a well-investigated model of human absence epilepsy) [30], in which it was demonstrated that exogenous ketone supplements (such as KE) delayed the onset of isoflurane-induced anesthesia (increased the time required before immobility) [23]. These effects may be clinically relevant because administration of exogenous ketone supplements-induced ketosis are more and more widely used as a metabolic therapy in the treatment of different CNS diseases, such as epilepsy or other seizure disorders [2,8,31–34]. Consequently, in order to implement a safe and successful anesthesia, potential effects of ketosis on the latency to anesthesia might need to be considered when epileptic patients are undergoing anesthetic

procedures. For this reason, this study was performed on WAG/Rij rats, to better understand the ketone supplement-evoked effects on isoflurane-generated onset of anesthesia (immobility) and its mechanism of action under epileptic condition.

In this study we hypothesized that adenosine receptor inhibition may modulate the exogenous ketone supplement-evoked delay in the latency to onset of immobility.

Methods

Animals

Animal treatments were carried out according to the Hungarian Act of Animal Care and Experimentation (1998, XXVIII, section 243), European Communities Council Directive 24 November 1986 (86/609/EEC) and EU Directive 2010/63/EU to use and treat animals in experimental laboratories. The experimental design was approved by the Animal Care and Experimentation Committee of the Eötvös Loránd University (Savaria University Centre) and National Scientific Ethical Committee on Animal Experimentation (Hungary) under license number VA/ÉBNTF02/85–8/2016.

Male WAG/Rij rats (n = 64; 6 months old, 315–330 g; breeding colony of WAG/Rij rats at Eötvös Loránd University, Savaria University Centre, Szombathely, Hungary) were kept in groups of 3–4 under standard laboratory conditions (12:12 h light-dark cycle, light was on from 08.00 AM to 08.00 PM; free access to food and water; air-conditioned room at 22 ± 2^{o}C). Rats were fed with standard rodent chow diet (SD), and received oral (intragastric) gavage of either water (control) or different ketone supplements (KE, KS, KSMCT, KEKS or KEMCT). The animals were euthanized in their home cage after the last treatment and data collection by using isoflurane. All efforts were made to minimize pain and suffering and to reduce the number of animals used.

Treatment groups and detection of immobility

Both KE (1,3-butanediol—acetoacetate diester) and KS (Na^+/K^+ - βHB mineral salt) were developed by D'Agostino et al. [2] (University of South Florida/USF, United States) in collaboration with Savind, Inc. (Urbana, IL, United States). Ketone salt was mixed into a 50% solution (375 mg/g pure βHB and 125 mg/g of Na^+/K^+ in a 1:1 ratio). Medium chain triglyceride (MCT) oil (pharmaceutical grade; approximately 60% caprylic triglyceride and 40% capric triglyceride) was purchased from Now Foods (Bloomingdale, IL, United States).

We demonstrated previously the tolerability and effectiveness of exogenous ketone supplements KE, KS, KSMCT (mix of KS and MCT oil in a 1:1 ratio), KEKS (mix of KE and KS in a 1:1 ratio) and KEMCT (mix of KE and MCT oil in a 1:1 ratio) given by intragastric gavage (*ad libitum* access to normal rat chow + 2.5 g/kg body weight supplements by gavage once/day in WAG/Rij rats) [1,4,5,31]. Mix of ketone supplements (KSMCT, KEKS, KEMCT) was carried out at the ELTE (Savaria University Centre, HUNGARY).

These types and dose of ketone supplements effectively induced and maintained ketosis in our previous studies [1,5,31] without causing side effects. Therefore, in the first phase of this study, 2.5 g/kg/day dosage of ketone supplements (KE, KS, KSMCT, KEKS and KEMCT) was administered daily by gavage for 7 days. In the second phase of the study, to investigate the putative adenosinergic mechanism of action of ketone supplements on isoflurane-evoked anesthesia (latency to immobility), we also used a specific A1R antagonist DPCPX and a selective A2A receptor antagonist SCH 58261, which drugs were dissolved in 10% dimethyl sulfoxide (DMSO). All drugs were purchased from Sigma-Aldrich Inc. (Hungary, Budapest). In order to minimize the putative adverse effects of drugs and to induce antagonism of A1Rs and A2ARs without changes in absence epileptic activity we used 0.2 mg/kg DPCPX and 0.5 mg/kg SCH 58261 in combination with KEKS (2.5 g/kg/day, gavage), because these doses alone did not change the SWD number in WAG/Rij rats [31,35, and unpublished, preliminary results]. Moreover, it was demonstrated previously that 10% of DMSO alone has no effect on SWD number [36].

To familiarize the animals to the methods, the 7 days gavage treatment was preceded by i.p. injection of 0.5 ml saline/100 g body weight and (30 min later) by water gavage for 5 days (adaptation period). Following adaptation period, rats were randomly assigned into 8 groups with 8 animals in each group. All of the rats were injected i.p. by 0.5 ml saline/100 g body weight/every day 30 min before gavage. After the i.p. saline injection, water (2.5 g/kg body weight/day, group 1; SD, control group) or exogenous ketone supplements (KE, KS, KSMCT, KEKS or KEMCT: 2.5 g/kg body weight/day; group 2—6, respectively) were administered by gavage for 7 days. One hour after the 7^{th} treatments, anesthesia was induced in an air tight anesthesia chamber with isoflurane (3% isoflurane gas mixed with air for 5 min). Immobility (time from chamber closure until end phase of anesthetic induction: immobility) was measured and analyzed by a blinded observer similar to previously [23]. Based on results on group 2 - 6, the most effective ketone supplement (KEKS) was chosen for investigation of the putative mechanism of action. Therefore, animals (group 7 and group 8) were i.p. injected by saline and gavaged for 7 days by KEKS similar to described above, but on the 7^{th} treatment (KEKS gavage) day, the i.p. injections contained 0.2 mg/kg DPCPX (group 7) or 0.5 mg/kg SCH 58261 (group 8). Finally, after combined administration of KEKS with i.p. DPCPX (group 7) or SCH58261 (group 8) anesthesia was induced and immobility was measured similar to group 2—6 on the 7^{th} treatment days. Each rat was used only in one of the treatment groups and was euthanized with isoflurane after the 7^{th} treatment and data collection.

Measurement of blood βHB and glucose levels as well as body weight

Blood was taken from the tail vein of rats. βHB levels were measured by a commercially available glucose and ketone monitoring system (Precision Xtra™, Abbott Laboratories, Abbott Park, IL, USA) [1,23,31]. Total blood ketone levels (D-HB + L-HB acetoacetate + acetone) would be higher than we measured because this instrument only measures blood levels of D-HB. Baseline HB and glucose levels were measured on the last (5^{th}) day of the adaptation period (group 1 - 6). Levels of HB and glucose were

measured again after the last (7^{th}) day of water (group 1, control) and ketone supplementation (gavage, group 2 - 6) on awake animals, approximately 10 min after the detection of isoflurane-induced immobility [23].

Body weight of rats were measured before (on last/5^{th} day of the adaptation period) and after (on last/7^{th} day of gavage) the treatments (group 1 - 6).

Statistics

All data were presented as the mean ± standard error of the mean (S. E. M.). We compared the latency of isoflurane-induced immobility in control group (SD; group 1; gavaged by water for 7 days) and treated groups (gavaged by different exogenous ketone supplements for 7 days: group 2 - 6; administration of KEKS in combination with DPCPX or SCH 58261 on 7^{th} treatment days: group 7 and group 8). Moreover, baseline (last/5^{th} day of the adaptation period; group 1 - 6), control (SD; group 1; 7^{th} day) and ketone supplements-induced (group 2—6; 7^{th} day) blood glucose and βHB levels as well as body weight (before treatment and after treatment: group 1—6) were also compared. Data analysis was performed using GraphPad Prism version 6.0a using a two-way ANOVA with Tukey's multiple comparisons test. Pearson correlation was calculated for blood βHB and anesthesia latency as individual data points and as group means [23]. Results were considered significant when $p < 0.05$.

Results

Effects of exogenous ketone supplements on blood βHB and glucose levels and body weight

A significant increase in blood βHB levels was demonstrated after the final (7^{th}) treatment by all exogenous ketone supplements (KE, KS, KSMCT, KEKS and KEMCT; group 2 - 6), compared to both control (SD; $p<0.01$ for KS; $p<0.001$ for KSMCT; $p<0.0001$ for KE, KEKS and KEMCT) and baseline ($p<0.001$ for KS; $p<0.0001$ for KE, KSMCT, KEKS and KEMCT) levels (Fig. 1A; Table 1).

After the 7^{th} treatment day, changes in glucose levels and body weight of animals were not detected (Fig. 1 B and C; Table 1 and Table 2).

Effect of exogenous ketone supplements on isoflurane-induced anesthesia: delay in the latency to onset of immobility

Treatments by all exogenous ketone supplements (KE, KS, KSMCT, KEKS and KEMCT; group 2 - 6) caused a significant increase in the number of seconds required before anesthetic induction (the time until immobility), compared to control (SD; $p<0.05$ for KSMCT; $p<0.001$ for KS; $p<0.0001$ for KE, KEKS and KEMCT) (Fig. 1D; Table 3) on the 7^{th} day of treatment.

Exogenous ketone supplement-induced delay in isoflurane-generated anesthesia (increase in latency to immobility) positively correlated with blood βHB levels when individual data points (R^2=0.2933) or the group means were considered (R^2=0.5553) (Fig. 1 E and F, respectively).

Effect of A1 and A2A receptor inhibition on KEKS-evoked increase in latency to immobility

It was demonstrated that i.p. 0.2 mg/kg DPCPX completely abolished the effect of KEKS on latency to immobility (group 7) (Fig. 1G), whereas i.p. 0.5 mg/kg SCH 58261 (group 8) was ineffective on the KEKS-induced effect (Fig. 1G; Table 3). After combined administration of KEKS with SCH 58261 on the 7th day of gavage, latency to immobility significantly increased, compared to control (SD; Fig. 1G) and both the rate of this increase and its significance level ($p<0.0001$) was similar to results, which were recorded after gavage of KEKS alone (group 5) (Fig. 1G).

Fig. 1. *Near here.*

Table 1. *Near here.*

Table 2. *Near here.*

Table 3. *Near here.*

Discussion

In this study we demonstrated that inhibition of A1Rs completely abolished the KEKS-evoked delay in isoflurane-induced anesthesia (immobility) in WAG/Rij rats. Moreover, we extended our previous results showing that not only gavage of KE and KS [23], but also KSMCT, KEKS and KEMCT are able to increase both the blood level of βHB and number of seconds required before anesthetic induction (immobility).

Although isoflurane has been used in patients for nearly 50 years [19], its mechanism of action remains largely unknown. In spite of that both behavioral and physiological differences in functioning of sleep and general anesthetics-induced sleep-like state were demonstrated (e.g., general anesthesia is not able to appear spontaneously), it was suggested that several brain areas, such as cerebral cortex and the hypothalamic nucleus ventrolateral preoptic area may participate in both processes [37-39]. It was hypothesized, that anesthetics, such as isoflurane may induce anesthesia through common endogenous arousal neural circuitry/sleep pathways [39,40].

Administration of exogenous ketone supplements by gavage and subsequent metabolism [17,41,42] increases levels of ketone bodies in the blood stream (ketosis) [1,2,4,5]. Ketone bodies, such as βHB may

enter into the brain through blood brain barrier and modulate different physiological and pathophysiological processes, such as sleep or seizures [7,8,12]. As ketosis (βHB) increases adenosine level [14] in the brain tissue and adenosine has a role in the sleep generation [27,28], enhanced level of βHB generated by ketone supplements may modulate naturally occurring sleep. Indeed, exogenous ketone supplement-generated ketosis may evoke a decrease in total sleep time through ventrolateral preoptic area [20,21,39]. Moreover, it has been demonstrated that level and metabolism of both ketone bodies [7,18,43], as well as adenosine and expression of adenosine receptors [44] are regionally different in the brain, which strengthen the modulatory role of ketone bodies and adenosine in processes such as sleep and sleep-like states. Ketosis-evoked increase in extracellular adenosine levels may change neuronal activity in different brain areas [22,44] implicated in sleep/sleep-like effects by its receptors. Increased level of adenosine was demonstrated during waking whereas adenosine concentration decreased during sleep in the brain [45]. Adenosine agonists induced sleep/electroencephalographic slow-wave activity, but adenosine receptor antagonists (e.g., a non-selective antagonist of adenosine receptors caffeine) reversed effects of adenosine on the sleep [46]. Moreover, adenosine accumulates under, for example, sleep deprivation and may have a role in the anesthetic action of isoflurane [27,39]: theophylline (a non-selective antagonist of adenosine receptors) reversed the cerebral effects of isoflurane in dogs (e.g., EEG has been changed from a sleep pattern to an awake pattern) [29] and caffeine accelerated emergence from isoflurane-evoked anesthesia in humans [47]. Moreover, enhanced activity of A1Rs (e.g., by an A1R agonist N-sulfophenyl adenosine) may cause increase in anesthesia recovery time [48] and isoflurane may activate A1Rs [49]. It has been demonstrated that receptors of adenosine, such as inhibitory A1Rs and excitatory A2ARs are expressed brain areas implicated in the generation of sleep and sleep-like effects, such as ventrolateral/lateral preoptic area and basal forebrain [28]. Thus, adenosine may be a link between the anesthetic actions of isoflurane and sleep regulation as an endogenous sleep factor. Both A1Rs and A2ARs are implicated in sleep generation, but A2ARs are considered more important in sleep regulation [28]: increased activity of A2ARs, for example, in ventrolateral/lateral preoptic area may induce sleep through sleep-active/promoting neurons [50,51]. However, our results suggest that A2ARs did not modulate the effect of exogenous ketone supplement-evoked ketosis on isoflurane-induced anesthesia (latency to immobility) (Fig. 1G). It was also demonstrated that inhibition or disinhibition by A1Rs (e.g., in wake-promoting neurons of basal forebrain or sleep-active neurons of ventrolateral preoptic area, respectively) may induce sleep [28,52,53]. Nevertheless, A1Rs may also promote wakefulness by inhibition of sleep-active neurons in lateral preoptic area [50]. Based on these results, we can hypothesize that adenosinergic system may modulate the influence of exogenous ketone supplement-generated ketosis on the onset of isoflurane-induced immobility by inhibition of sleep active neurons (possibly through A1Rs), which processes lead to delay in the anesthetic effects of isoflurane. Moreover, modulatory effects of adenosine receptor antagonists and an A1R agonist on isoflurane-induced anesthetic effects and on emergence from anesthesia [29,47,48] were also demonstrated. Thus, it is possible that exogenous ketone supplement-induced ketosis not only delay the onset of isoflurane induced anesthesia (immobility), but also modulate the time required for recovery from anesthesia. However, further studies are needed to determine the exact effect and

mechanism(s) of action of exogenous ketone supplement-evoked ketosis on isoflurane-generated anesthetic effects.

It has been demonstrated that gavage of exogenous ketone supplements, such as KSMCT for 7 days not only increases the number of seconds required before isoflurane-induced anesthetic induction (the time until immobility) (Fig. 1D) [23], but also generates decrease in both anxiety level on elevated plus maze [54] and absence epileptic activity [31] in WAG/Rij rats. These effects may be in correlation with enhanced level of βHB (Fig. 1E and F) [23,31,54]. Moreover, it was showed that inhibition of A1Rs may abolish the anti-anesthetic (Fig. 1G), antiepileptic [31] and anxiolytic [54] effects of exogenous ketone supplements, suggesting that adenosinergic system may modulate the ketone supplements (ketosis) induced influences in the CNS. Indeed, it was proposed that adenosinergic system (mainly through A1Rs) has a role in the modulation of sleep and sleep-like effects [27-29], different types of epilepsies [55-57] and anxiety [58-60]. However, new studies are needed to reveal the likely (at least partly) common mechanism(s), as well as interactions of adenosine receptors and adenosine receptor-evoked changes (e.g., in ion channels, signal transduction, metabolic processes) in different brain areas involved in sleep/sleep-like effects, epilepsy and anxiety, by which ketone supplements could exert its above mentioned influences.

One limitation of our study is that we used the WAG/Rij rat strain exclusively to investigate the effect of ketone supplementation on isoflurane-induced anesthesia. In addition, during this study we narrowed our focus on the influence of ketone supplement-evoked effects to the adenosinergic system. Nevertheless, this WAG/Rij rat strain is extensively used for investigation of different drugs on CNS diseases [1,61-64], and the present study further supports our previous experiments [23] on the role of the adenosinergic system. It has been suggested that the ketosis-evoked increase in adenosine levels [14] can modulate influence of ketone supplements not only on different CNS diseases [8,31,54], but also sleep and sleep-like effects [20,21,27-29] *via* adenosinergic system (likely through A1Rs). Consequently, we propose that the adenosinergic system may be one of main neurotransmitter systems by which ketone supplements can exert their influence on isoflurane-induced anesthesia.

Conclusion

The present study strengthened the putative clinical and surgical relevance of ketosis-evoked influences on sleep and sleep-like effects suggested by our previous results: exogenous ketone supplement-evoked nutritional ketosis may increase the resistance to the isoflurane-induced anesthetic influence by delaying the onset of anesthesia (the time until immobility). Thus, monitoring of blood ketone levels in humans undergoing isoflurane and (theoretically) other inhalational anesthesia may be important and helpful for the anesthesiologists. Moreover, inhibitory effect of DPCPX on KEKS-evoked delay in isoflurane-generated anesthesia suggests that the adenosinergic system, likely *via* A_1Rs, may modulate the exogenous ketone supplements-evoked anti-anesthetic influence. However, further studies are needed to reveal the exact mechanism(s) of action of exogenous ketone supplements on isoflurane-generated anesthesia not only

in animals, but also in human subjects because ketosis (evoked by ketone supplements used in normal and pathological conditions) may modify the time needed for anesthesia.

Abbreviations

A_1R: adenosine A_1 receptor; A2AR: adenosine A2A receptor; βHB: beta-hydroxybutyrate; CNS: central nervous system; DMSO: dimethyl sulfoxide; DPCPX: 1,3-dipropyl–8-cyclopentylxanthine; i.p.: intraperitoneal; K_{ATP} channels: ATP-sensitive potassium channels; KE (ketone ester): 1,3 butanediol-acetoacetate diester; KEKS (mix of KE and KS); KEMCT (mix of KE and MCT oil); KS: ketone salt; KSMCT (mix of KS and MCT oil); MCT: medium chain triglyceride; SCH 58261: 7-(2-phenylethyl)–5-amino–2-(2-furyl)-pyrazolo-[4,3-e]–1,2,4-triazolo[1,5-c]pyrimidine; SD: standard rodent chow diet; WAG/Rij: Wistar Albino Glaxo/Rijswijk

Declarations

Acknowledgements

Not Applicable

Funding

This work was supported by the National Development Agency of Hungary (under Grant No. TIOP-1.3.1.-07/2-2F-2009-2008), OTKA K124558 Research Grant (to Zsolt Kovács), ONR Grant N000141310062 (to Dominic P. D'Agostino). The funding body had no influence on the design of the study, collection and analysis of data, interpretation of results and writing the manuscript.

Availability of data and materials

The data used and/or analyzed during the current study available from the corresponding author on reasonable request.

Authors' Contributions

All authors read and approved the final manuscript.

ZK: design of experiments, acquisition, analysis, and interpretation of data, writing manuscript, BB: acquisition of data, DPD: revising manuscript, CA: analysis of data, writing manuscript

Ethics approval and consent to participate

Animal treatments were carried out according to Hungarian Act of Animal Care and Experimentation (1998, XXVIII, section 243), European Communities Council Directive 24 November 1986 (86/609/EEC) and EU Directive 2010/63/EU to use and treat animals in experimental laboratories. The experimental design was approved by the Animal Care and Experimentation Committee of the Eötvös Loránd University (Savaria University Centre) and National Scientific Ethical Committee on Animal Experimentation (Hungary) under license number VA/ÉBNTF02/85-8/2016.

Consent for publication

Not Applicable

Competing interests

International Patent # PCT/US2014/031237, University of South Florida for DPD: “Compositions and Methods for Producing Elevated and Sustained Ketosis”. Non provisional patent No. 210112-9018-US02 for AC and DPD. Technology Title: “Methods of Increasing Latency of Anesthetic Induction Using Ketone Supplementation”. All authors declare that there are no additional conflicts of interest.

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Tables

	Blood βHB level			
Treatments (2.5 g/kg/day; Fig. 1A)	**Baseline**	**7th treatment day**		
	mmol/l (mean ± S.E.M.)	mmol/l (mean ± S.E.M.)	Compared to baseline (significance level/q-value)	Compared to control (significance level/q-value)
SD (control; group 1)	0.80±0.037	0.86±0.018	-/0.945	-
KE (group 2)	0.79±0.029	1.76±0.087	****/14.550	****/13.610
KS (group 3)	0.83±0.031	1.25±0.033	***/6.805	**/5.860
KSMCT (group 4)	0.81±0.023	1.33±0.049	****/7.939	***/6.994
KEKS (group 5)	0.83±0.041	1.55±0.073	****/11.340	****/10.400
KEMCT (group 6)	0.80±0.033	2.14±0.172	****/20.230	****/19.280

	Blood glucose level			
Treatments (2.5 g/kg/day; Fig. 1B)	**Baseline**	**7th treatment day**		
	mg/dl (mean ± S.E.M.)	mg/dl (mean ± S.E.M.)	Compared to baseline (significance level/q-value)	Compared to control (significance level/q-value)
SD (control; group 1)	89.00±1.937	95.38±2.672	-/1.764	-
KE (group 2)	91.50±2.284	97.63±4.342	-/2.386	-/0.623
KS (group 3)	88.38±1.772	100.13±5.482	-/3.078	-/1.314
KSMCT (group 4)	83.63±3.600	97.00±5.745	-/2.213	-/0.449
KEKS (group 5)	86.38±2.398	92.13±3.388	-/0.865	-/0.899
KEMCT (group 6)	85.00±2.659	80.88±4.286	-/2.248	-/4.011

Table 1 Effect of ketone supplements on blood βHB and glucose levels on the 7th day of gavage. Abbreviations: KE, ketone ester; KEKS, mix of KE and KS in a 1:1 ratio; KEMCT, mix of KE and medium chain triglyceride (MCT) oil in a 1:1 ratio; KS, ketone salt; KSMCT, mix of KS and MCT oil in a 1:1 ratio; SD, standard diet/control; **p<0.01; ***p<0.001; ****p<0.0001.

	Body weight		
Treatments (2.5 g/kg/day; Fig. 1C)	**Before the treatments**	**After the treatments**	
	Gram (mean ± S.E.M.)	Gram (mean ± S.E.M.)	Compared to 'Before the treatments' (significance level/q-value)
SD (control, group 1)	325.0±3.134	331.8±4.427	-/1.464
KE (group 2)	331.9±2.496	324.9±2.608	-/0.027
KS (group 3)	323.1±2.394	313.5±3.942	-/2.495
KSMCT (group 4)	318.0±5.119	314.0±5.719	-/2.386
KEKS (group 5)	316.9±5.377	306.6±6.434	-/3.986
KEMCT (group 6)	321.8±5.502	316.8±5.640	-/1.790

Table 2 Effect of ketone supplements on body weight. Abbreviations: KE, ketone ester; KEKS, mix of KE and KS in a 1:1 ratio; KEMCT, mix of KE and medium chain triglyceride (MCT) oil in a 1:1 ratio; KS, ketone salt; KSMCT, mix of KS and MCT oil in a 1:1 ratio; SD, standard diet/control.

	Latency to immobility	
Treatments (2.5 g/kg/day; Fig. 1D and G)	sec (mean ± S.E.M.)	Compared to control (significance level/q-value)
SD (control, group 1)	142.88±2.474	-
KE (group 2)	173.50±3.105	****/10.220
KS (group 3)	162.50±1.679	***/6.548
KSMCT (group 4)	157.88±3.446	*/5.005
KEKS (group 5)	183.88±4.299	****/13.680
KEMCT (group 6)	171.75±2.226	****/9.634
K+DPCPX (group 7)	138.13±4.202	-/1.177
K+SCH (group 8)	187.13±5.531	****/12.290

Table 3 Effect of ketone supplements alone and after combined application of KEKS with DPCPX and SCH 58261 on latency to immobility on the 7th day of gavage. Abbreviations: DPCPX, 1,3-dipropyl-8-cyclopentylxanthine (a specific adenosine A1 receptor/A1R antagonist); KE, ketone ester; KEKS, mix of KE and KS in a 1:1 ratio; KEMCT, mix of KE and medium chain triglyceride (MCT) oil in a 1:1 ratio; KS, ketone salt; KSMCT, mix of KS and MCT oil in a 1:1 ratio; K+DPCPX, combined administration of KEKS (K; gavage) with i.p. 0.2 mg/kg DPCPX; K+SCH, combined administration of KEKS (K; gavage) with i.p. 0.5 mg/kg SCH 58261 (SCH); SD, standard diet/control; SCH, SCH 58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (a selective adenosine A2A receptor/A2AR antagonist); *p<0.05; ***p<0.001; ****p<0.0001.

Figures

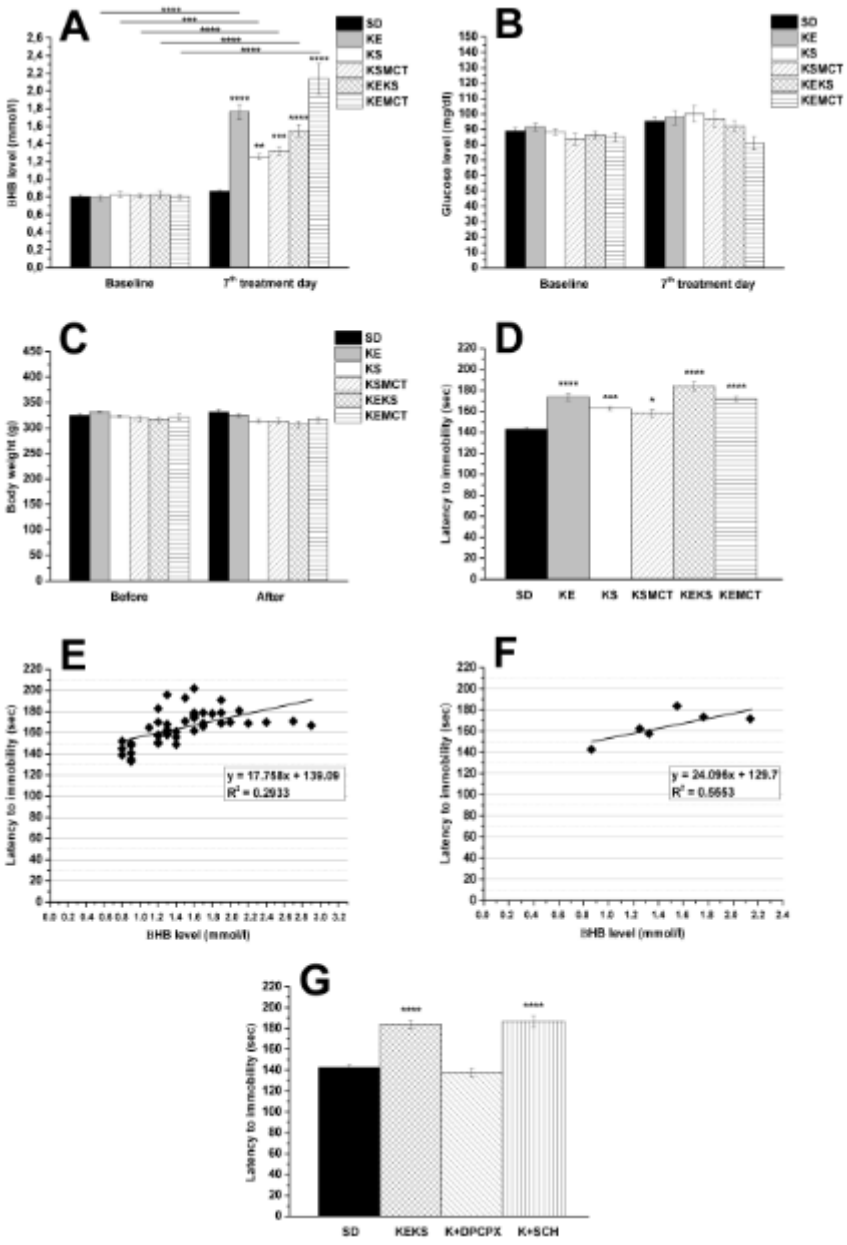

Figure 1

Ketone supplement-evoked delay in the onset of isoflurane-induced anesthesia in WAG/Rij rats; A. Blood βHB levels were significantly elevated in all groups (group 2 – 6) gavaged by ketone supplements (KE, KS, KSMCT, KEKS and KEMCT) for 7 days, compared to both control (standard diet/SD, gavaged with water for 7 days) and to their baseline; B and C. After different treatments, glucose level (B) and body weight (C) of animals did not change compared to both control (SD) and to their baseline; D. Gavage by exogenous ketone supplements (KE, KS, KSMCT, KEKS and KEMCT; group 2 - 6) significantly increased the latency to anesthetic induction (the time until immobility), compared to control (SD) on the 7th day of gavage; E and F. There was a positive correlation between latency to immobility and blood βHB levels when all data point (E; R2=0.2933) or the group means (F; R2=0.5553) were considered; G. Combined administration of DPCPX (i.p. 0.2 mg/kg) with KEKS (K+DPCPX) completely abolished the effect of KEKS on latency to immobility (group 7), whereas SCH 58261 (i.p. 0.5 mg/kg) was ineffective on the KEKS-induced effect

(K+SCH; group 8); Abbreviations: After, after the treatments; Before, before the treatments; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine (a specific adenosine A1 receptor/A1R antagonist); KE, ketone ester; KEKS, mix of KE and KS in a 1:1 ratio; KEMCT, mix of KE and medium chain triglyceride (MCT) oil in a 1:1 ratio; KS, ketone salt; KSMCT, mix of KS and MCT oil in a 1:1 ratio; K+DPCPX, combined administration of KEKS (K; gavage) with i.p. 0.2 mg/kg DPCPX; K+SCH, combined administration of KEKS (K; gavage) with i.p. 0.5 mg/kg SCH 58261 (SCH); SD, standard diet/control; SCH 58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (a selective adenosine A2A receptor/A2AR antagonist); *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

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