KD-64 – a new selective A_{2A} adenosine receptor antagonist has anti-inflammatory activity but contrary to the non-selective antagonist – caffeine does not reduce diet-induced obesity in mice

Magdalena Kotańska[1*], Anna Dziubina[2], Małgorzata Szafarz[3], Kamil Mika[1], Karolina Reguła[1], Marek Bednarski[1], Małgorzata Zygmunt[1], Anna Drabczyńska[4], Jacek Sapa[1], Katarzyna Kieć-Kononowicz[4]

[1]Department of Pharmacological Screening, Jagiellonian University Medical College, 9 Medyczna Street, PL 30-688 Krakow, Poland

[2]Department of Pharmacodynamics, Jagiellonian University Medical College, 9 Medyczna Street, PL 30-688 Krakow, Poland

[3]Department of Pharmacokinetics and Physical Pharmacy, Jagiellonian University Medical College, Krakow, Poland

[4]Department of Technology and Biotechnology of Drugs, Faculty of Pharmacy, Jagiellonian University Medical College, Krakow, Poland

*** Correspondence:** Magdalena Kotańska, e-mail: magda.dudek@uj.edu.pl

Tel.: +48 12 6205530; fax: +48 12 6205530

All authors contributed to this work.

Running title: Some properties of KD-64 - selective A_{2A} adenosine receptor antagonist

Abstract

The A_2 adenosine receptors play an important role, among others, in the regulation of inflammatory process and glucose homeostasis in diabetes and obesity. Thus, the presented project evaluated of influence of the selective antagonist of A_{2A} adenosine receptor – KD-64 as compared to the known non-selective antagonist – caffeine on these two particular processes. Two different inflammation models were induced namely local and systemic inflammation. Obesity was induced in mice by high-fat diet and the tested compounds (KD-64 and caffeine) were administrated for 21 days. KD-64 showed anti-inflammatory effect in both tested inflammation models and administered at the same dose as ketoprofen exerted stronger effect than this reference compound. Elevated levels of IL-6 and TNF-α observed in obese control mice were significantly lowered by the administration of KD-64 and were similar to the values observed in control non-obese mice. Interestingly, caffeine increased the levels of these parameters. In contrast to caffeine which had no influence on AlaT activity, KD-64 administration significantly lowered AlaT activity in the obese mice. Although, contrary to caffeine, KD-64 did not reduce diet-induced obesity in mice, it improved glucose tolerance. Thus, the activity of the selective adenosine A_{2A} receptor antagonist was quite different from that of the non-selective.

Keywords: selective A_{2A} adenosine receptor antagonist, caffeine, obesity, inflammation, vascular permeability, interleukine-6

1. Introduction

Obesity is defined as over-storage of lipids in adipose tissue that occurs when the amount of supplied energy significantly exceeds its consumption by the body [1]. Currently, it is an extremely important civilization problem, since obesity is considered a risk factor for cardiovascular diseases (e.g. ischemic heart disease, hypertension, heart failure, stroke), diabetes, dyslipidemia, autoimmune diseases and even cancer [2]. The reasons for overweight (and obesity) are numerous and varied. Genetic, environmental and/or social factors as well as the hormonal status of the body play the major role in its pathogenesis [3]. According to the current theory, development of obesity, associated with adipocyte hypertrophy and hyperplasia, is connected not only with disturbances in the secretory function of the fat tissue, but also with increased inflammatory activation of adipocytes, release of pro-inflammatory cytokines and dysregulation of adipokine secretion [4 ,5 ,6 ,7]. An increase in the levels of pro-inflammatory cytokines and proteins such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), leptin or C-reactive protein (CRP) in both blood and adipose tissue itself was observed as obesity progressed. On the other hand, reduced levels of anti-inflammatory factors for instance adiponectin [8] as well as various changes (increase or decrease) in IL-10 levels together with the mostly unchanged levels of IL-8 [9, 10] seem to be also relevant. Until now, the mechanism triggering inflammatory activation of adipose tissue has not been clearly defined. Different theories point to the role of hypoxia of adipocytes [11], cellular stress during obesity development [12], and elevated glucose level, all generating large amounts of free oxygen radicals in adipocytes and stimulating pro-inflammatory cytokines secretion [13, 14].

Pharmacological treatment of obesity remains an unresolved problem. The role of adenosine receptor signalling in the development and progression of numerous diseases has been emphasized for years. Adenosine is an endogenous purine nucleoside that participates in the

development of obesity [15]. It works through adenosine receptors such as A_1, A_{2A}, A_{2B} and A_3 that differ in their pharmacological profile (including affinity for adenosine), tissue localization and the system of second order messengers. Adenosine receptors are widely expressed in organs and tissues involved in metabolism regulation such as liver, pancreas, adipose tissue and muscles, moreover its presence on immune cells [16], points to their significant role in the inflammatory processes. Since all of these receptors are engaged in glucose homeostasis, adipogenesis, insulin resistance, inflammation and thermogenesis, treatment with specific agonist and/or antagonists could normalize several mechanisms involved in pathophysiology of obesity [17].

The A_{2A} receptor being the most abundant adenosine receptor in human and murine white and brown adipose tissue, as well as on immune cells and organs has become a potential target for obesity studies [16, 18]. Data indicate that adenosine signalling via A_{2A} is required for activation of brown adipose tissue and protects mice from diet-induced obesity [19]. It has been also demonstrated that A_{2A} receptor knockout mice exhibit impaired thermogenesis, oxygen consumption, and lipolysis [20]. Moreover, Csóka et al., (2017) observed the reduced food intake in such mice and consequently a lower body mass as compared to control animals [21]. A strong functional interaction between the dopamine D_2 and adenosine A_{2A} receptors [22] has been further discovered and a blockade of adenosine A_{2A} receptor can even mimic the action of D_2 agonists [23]. Since dopamine is known to be an important regulator of energy expenditure [24, 25] and food intake [26] reduced dopamine signal transduction may give rise to overeating and decreased energy consumption, both of which contribute to the positive energy balance seen in obesity [27].

The adenosine A_{2A} receptor is predominantly expressed on inflammatory cells, including neutrophils, mast cells, macrophages, monocytes, and platelets [28] and in many animal studies it has been demonstrated that its activation reduced inflammatory processes [28, 29]

and improved molecular markers of inflammation. On the other hand, it has been shown that the direct local injection of the selective A_{2A} receptor antagonist ZM 241385 in carrageenan-induced inflammatory hyperalgesia reduced inflammatory hypersensitivity suggesting that activation of peripheral adenosine A_{2A} receptors during inflammation is associated with mechanical hyperalgesia [30, 31] presented the series of A_{2A} adenosine receptor ligands with significant anti-inflammatory activity in carrageenan-induced paw edema model. These compounds have similar structure to KD-64 – ligand we have used in the presented study.

For comparison purposes caffeine was chosen, a non-selective A_1 and A_{2A} adenosine antagonist and the most popular and well-studied methylxantine which has been reported as thermogenic and lipolysis stimulator leading to fat oxidation in adipocytes and release of glycerol and fatty acids to the bloodstream [32, 33]. Moreover, caffeine modulates glucose metabolism and increases energy expenditure, as well as has impact on [34] body fat reduction [35] and weight loss [36] mostly as adjuwant agent [37, 38] especially during physical exercise or when administered simultaneously with the calorie restriction diets [39 ,40].

While non-selective adenosine A_1 and A_{2A} receptor antagonists such as caffeine are well investigated in obesity-related mechanisms, the results of studies on selective A_{2A} receptor antagonists are still inconsistent, therefore we have chosen for our experiments a selective A_{2A} receptor antagonist, designated as KD-64, with significant selectivity over other adenosine receptors (K_i [μM] values are 0.24, > 25, > 10 and > 10 for A_{2A}, A_1, A_{2B} and A_3 receptors, respectively) [41].

In the first part of our research we have estimated the effect of KD-64 on the inflammation in the carrageenan or zymosan induced models of inflammation. Further, in a diet-induced mice obesity model, we have confirmed the development of inflammation, and compared the

effects of investigated compound and caffeine, i.e. selective and non-selective A_{2A} receptor antagonists, on the primary metabolic variables.

2. Materials and Methods

2.1. Animals

Adult male Albino Swiss mice, CD-1, weighing 25–30 g were used in the inflammatory models and estimation of locomotor activity and adult female Albino Swiss mice, CD-1, weighing 19–22 g were used in the model of obesity. Animals were kept in environmentally controlled rooms, in standard cages lit by an artificial light for 12h each day. They had free access to food and water, except for the time of the acute experiment. The randomly established experimental groups consisted of 8 mice. All animal care and experimental procedures were carried out in accordance with European Union and Polish legislation acts concerning animal experimentation, and were approved by the Local Ethics Committee at the Jagiellonian University in Cracow, Poland (Permission No: 256/2015 and 55/2017).

2.2. Drugs, chemical reagents and other materials

Ketoprofen was used as standard anti-inflammatory compound and was purchased from Sigma-Aldrich (Poland). Carrageen was purchased from FCM Corporation (USA), Zymozan A and Evans blue from Sigma-Aldrich (Poland), Caffeine (used as standard in obese model) was purchased from Alfa-Aesar (Poland). KD-64 (Figure 1) [42] was synthesized in the Department of Technology and Biotechnology of Drugs, Faculty of Pharmacy, Jagiellonian University Medical College, Cracow, Poland. NMR and LC-MS techniques assessed identity and purity of final product. In all experiments ketoprofen, caffeine or KD-64 were administered as suspensions in 1% Tween 80.

Fig 1. Chemical structure of compound KD-64

2.3. Inflammation models

150 *2.3.1. Carrageenan-Induced Edema Model*

151 To induce inflammation, 0.1 ml of 1% carrageenan solution in water was injected into the
152 hind paw subplantar tissue of mice, according to the modified method of C. A. Winter and P.
153 Lence [43, 44], as described previously [45]. The development of paw edema was measured
154 with a plethysmometr (Plethysmometr 7140, Ugo Basile). Prior to the administration of the
155 tested substances (KD-64 or ketoprofen), paw diameters were measured and data were
156 recorded for further comparison. The KD-64 compound was administered at the doses of 1, 5
157 or 10 mg/kg, intraperitoneal (*ip*), prior to carrageenan injection, similarly ketoprofen
158 (reference standard) was administered at the dose of 5 mg/kg [46]. 1% Tween 80 (vehicle)
159 was administered by the same route to the control group (it had no effect on edema, data not
160 shown). Results were presented as changes in the hind paw volume 3 h after carrageenan
161 administration. Immediately, after measurement mice were administrated 2500 units/mice of
162 heparin *ip* and 20 minutes later sacrificed by decapitation. Blood was collected to the
163 Eppendorf tubes and centrifuged at 600 x g (15 min, 4°C) in order to obtain plasma used for
164 the determination of CRP levels.

165 *2.3.2. Zymosan A induced peritoneal inflammation*

166 Peritoneal inflammation was induced as described previously [47]. Zymosan A was freshly
167 prepared (2 mg/ml) in sterile 0.9% NaCl and 30 minutes after *ip* injection of the investigated
168 compounds (KD-64 or ketoprofen at the dose of 5 mg/kg), zymosan A was injected via the
169 same route. Four hours later the animals were killed by decapitation and blood was collected
170 into a heparin-containing tubes. After centrifugation mice plasma was stored for the further
171 measurements of the CRP levels. The peritoneal cavity was lavaged with 1.5 ml of PBS and
172 after 30s of gentle manual massaging the exudates were retrieved. Cells were counted using
173 an optical microscope (DM1000, Leica) and Bürker hemocytometer following staining with
174 Turk's solution.

175 *2.3.3. Vascular permeability*

176 In the control studies Evans blue was suspended in the saline (10 mg/ml) and injected
177 intravenously (*iv*) into the caudal vein, which was immediately followed by *ip* injection of
178 zymosan A. Thirty minutes later the animals were killed by decapitation and their peritoneal
179 cavities were lavaged with 1.5 ml of saline as described above. The lavage fluid was
180 centrifuged and the absorbance of the supernatant was measured at 620 nm in order to assess
181 vascular permeability by peritoneal leakage of *iv* injected Evans blue as described previously
182 [47]. In the subsequent experiments KD-64, ketoprofen (reference standard) both at the dose
183 of 5 mg/kg or vehicle (administered *sc*, control group) were injected *ip* 30 min before Evans
184 blue and zymosan A. Further procedures were the same as in the control group.

185 *2.4. Locomotor activity*

186 The locomotor activity was recorded with an Opto M3 multichannel activity monitor
187 (MultiDevice Software v1.3, Columbus Instruments, USA). It was evaluated as the distance
188 travelled by the animals while attempting to climb upward [48]. After *ip* administration of
189 KD-64 compound at the doses of 1, 5 or 10 mg/kg, each mouse was placed in a cage for a 30
190 minutes habituation period. After that time the number of crossings of photo beams was
191 measured for 20 minutes.

192 *2.5. Obesity study*

193 *2.5.1. Metabolic disturbance induced with a high-fat/sucrose diet and its influence on body*
194 *weight and spontaneous activity*

195 Mice were fed on high-fat diet consisting of 40% fat blend (Labofeed B with 40% lard,
196 Morawski Feed Manufacturer, Poland) for 15 weeks, water and 30% sucrose solution were
197 available *ad libitum* [49, 50]. Control mice were fed on a standard diet (Labofeed B,
198 Morawski Feed Manufacturer, Poland) and drank water only. After 12 weeks, mice with
199 obesity were randomly divided into three equal groups that had the same mean body weight

and were treated *ip* with tested compounds at the following doses: KD-64 5 mg/kg b.w./day or caffeine 50 mg/kg b.w./day and control group: 1% Tween 80 (vehicle) 0.35 ml/kg (fat/sugar diet + vehicle = obesity control group) once daily between 9:00 and 10:00 AM for 21 days. Control mice (control without obesity) were maintained on a standard diet, with *ip* administration of 1% Tween 80, 0.35 ml/kg (standard diet + vehicle = control group). Animals always had free access to feed, water and sucrose. After experiment mice were killed by decapitation and plasma was harvested to determine levels of TNF-α, IL-6 and activity of alanine aminotransferase (AlaT).

High-fat feed composition (932 g of dry mass): protein – 193 g, fat (lard) – 408 g, fiber – 28.1 g, crude ash – 43.6 g, calcium – 9.43 g, phosphorus – 5.99 g, sodium – 1.76 g, sugar – 76 g, magnesium – 1.72 g, potassium – 7.62 g, manganese – 48.7 mg, iodine – 0.216 mg, copper – 10.8 mg, iron – 125 mg, zinc – 61.3 mg, cobalt – 0.253 mg, selenium – 0.304 mg, vitamin A – 15000 units, vitamin D3 – 1000 units, vitamin E – 95.3 mg, vitamin K3 - 3.0 mg, vitamin B1 – 8.06 mg, vitamin B2 – 6.47 mg, vitamin B6 – 10.3 mg, vitamin B12 – 0.051 mg, folic acid – 2.05 mg, nicotinic acid – 73.8 mg, pantothenic acid – 19.4 mg, choline – 1578 mg.

The high-fat diet contained 550 kcal and the standard diet 280 kcal per 100 g.

The spontaneous activity of mice was measured on the 1st and 21st day of the treatment with a special RFID-system – TraffiCage (TSE-Systems, Germany). The animals were subcutaneously implanted with a radio-frequency identificator (RFID), which enabled to count the presence and time spent in different areas of the cage. The obtained data was grouped using an appropriate computer program [48].

2.6. Biochemical analysis

2.6.1. Glucose tolerance test

The glucose tolerance test was performed at the beginning of 16th week. After twenty administrations of the tested compounds (KD-64 or caffeine), food and sucrose were

discontinued for 20h and then glucose tolerance was tested. Glucose (1g/kg b.w.) was administrated *ip* [49, 50] and blood samples were taken from the tail vein at the time points: 0 (before glucose administration), 30, 60 and 120 minutes after administration. Glucose levels were measured with glucometer (ContourTS, Bayer, Germany, test stripes: ContourTS, Ascensia Diabetes care Poland Sp. z o.o., Poland, REF:84239666). The area under the curve (AUC) was calculated using the trapezoidal rule.

2.6.2. Insulin tolerance test

Insulin tolerance was tested on the next day after the glucose tolerance test. Mice had free access to standard food and water, but 3h before insulin tolerance test the food was taken away. Insulin (0.5 IU/kg b.w.) was injected *ip* and blood samples were collected at the time points: 0, 15 and 30 minutes from the tail vein and glucose levels were measured with glucometer (ContourTS, Bayer, Germany, test stripes: ContourTS, Ascensia Diabetes care Poland Sp. z o.o., Poland, REF:84239666) [49, 50]. The AUC was calculated using the trapezoidal rule.

2.6.3. Plasma levels of IL-6, TNF-α, CRP and AlaT activity

On the next day after insulin tolerance test, 20 minutes after *ip* administration of heparin (2500 units/mice) animals were sacrificed by decapitation. The blood was collected and then centrifuged at 600 x g (15 min, 4°C) in order to obtain plasma. To determine AlaT activity in the plasma samples, standard enzymatic spectrophotometric test (Biomaxima S.A. Lublin, Poland, catalogue number: 1-023-0150) was used. In order to quantify IL-6 and TNF-α LANCE® Ultra Detection Kits (PerkinElmer, Inc, USA, catalogue numbers: TRF1505, TRF1504C/TRF1504M) were used. For the determination of CRP standard enzymatic spectrophotometric tests (Shanghai Sunred Biological Technology Co., Ltd, China, catalogue number: 201-02-0219) were applied.

2.6. Statistical analysis

The obtained results were analyzed using a one-way variance analysis (ANOVA), followed by a Dunnett post-hoc test, with the significance level set at 0.05 (locomotor activity, AlaT activity, IL-6 or TNF-α levels,), a two-way variance analysis (ANOVA), followed by a Bonferroni post-hoc test (changes in body weight) or a Multi-t test (glucose tolerance test, insulin tolerance test, spontaneous activity). The results were expressed as the means ± standard error of the mean (SEM). Graph Pad Prism 6.0 was used for data analysis.

3. Results

3.1. Influence of KD-64 on spontaneous activity

Compound KD-64 did not affect spontaneous activity in mice after single intraperitoneal administration at all tested doses. The results are shown in Figure 2.

Fig 2. Locomotor activity after a single administration of KD-64

Results are mean ± SEM, n=6. Comparisons were performed using one-way ANOVA Dunnet's post hoc test.

3.2. Influence of KD-64 on carrageenan-induced paw edema in mice

The mouse paw became edematous after the injection of carrageenan, and in the control group edema reached a peak at 3h (increase by 97.7% of the initial volume). The increase in paw edema was significantly inhibited by the KD-64 administration at a dose of 5 mg/kg b.w. as compared to the control group, which was given carrageenan and vehicle only (Figure 3A). Since the results were comparable to the ones observed in the group receiving ketoprofen, 5 mg/kg b.w. of KD-64 (as an active dose) has been selected for further studies. In the plasma of mice treated with KD-64 significant decrease in CRP level (similar to the one observed after ketoprofen administration) was also determined (Figure 3B).

Fig 3. Anti-inflammatory effects of the tested compounds in the carrageenan-induced paw edema test

(**A**) Changes in the paw volume in 3h after drug administration in relation to the initial volume (before carrageenan injection). Results are mean ± SEM, n=8. Comparisons were performed using one-way ANOVA Dunnet's post hoc test. * Significant against control mice administered carrageenen; *p<0.05. (**B**) Concentration of C-reactive protein in plasma. Results are mean ± SEM, n=8. Comparisons were performed by t-Student test. * Significant against control mice, ^ Significant against control mice administered carrageenen; *p<0.05, ^^p<0.01, ^^^p<0.001.

3.3. Influence of KD-64 on Zymosan A induced peritoneal inflammation and vascular permeability

The early infiltration of neutrophils measured at 4h after zymosan-induced peritonitis was significantly inhibited in the group receiving KD-64 at the dose of 5 mg/ kg b.w. as compared to the control group, which was given zymosan A alone and leucocytosis was comparable to the one measured after ketoprofen administration (Figure 4A). CRP concentration in mice plasma was also decreased in the group receiving KD-64, interestingly levels of CRP after ketoprofen administration did not change significantly (Figure 4B). The early vascular permeability measured at 30 min after zymosan-induced peritonitis was significantly inhibited in the group receiving KD-64 compared to the control group, which was given zymosan A alone (Figure 4C). After ketoprofen administration vascular permeability also decreased however it was still significantly higher than in control group without induced peritoneal inflammation. Thus, in both experiments KD-64 administered at the same dose as ketoprofen exerted stronger effect than reference compound.

Fig 4. Anti-inflammatory effects of the tested compounds in model of zymosan-induced peritonitis in mice

(**A**) Neutrophil infiltration during zymosan-induced peritonitis in mice, (**B**) Concentration of C-reactive protein in plasma, (**C**) Vascular permeability. Results are mean ± SEM, n=8. Comparisons were performed by t-Student test. * Significant against control mice, ^ Significant against control mice administered zymosan. *,^p<0.05, **,^^p<0.01, ***p<0.001.

3.4. Influence of KD-64 on body weight and peritoneal fat

Mice fed with high-fat/sugar diet showed more weight gain throughout the 12-week period of inducing obesity as compared to the control group. Animals fed with high-fat diet and treated with KD-64 at the dose of 5 mg/kg b.w. showed significantly less weight gain than mice from the obese control group, however only during the first week of administration. From the second week of KD-64 administration the difference wasn't significant. Mice from the group receiving caffeine (50 mg/kg b.w./day, *ip*) starting from the first week of treatment gained less weight compared to the control group and at the end of the experiment weighed significantly less. The results are shown in Figures 5A and 5B. Animals consuming high-fat feed had also significantly higher amount of fat in peritonea. The results are shown in Figure 5C.

Fig 5. Effect of administration of KD-64 or caffeine on body weight and mass of adipose pads

(**A**) Percent change of body weight during the administration. (**B**) Sum of weight changes. (**C**) Mass of adipose pads. Results are expressed as means ± SEM, n=8. Multiple comparisons were performed by two-way ANOVA, Bonferroni's post hoc (**A**) or one-way ANOVA Dunnet's post hoc tests (**B**, **C**). ^ Significant against control mice fed fat/sugar diet; * Significant against control mice fed standard diet; ^p<0.05, ^^p<0.01, ***,^^^p<0.001.

3.5. Influence of KD-64 on plasma IL-6 and TNF-α levels in obese mice

In obese control mice higher plasma levels of IL-6 and TNF-α were observed than in control standard fed mice. However, they were significantly lowered by the administration of KD-64 for 21 days at the dose of 5 mg/kg b.w./day and were similar to the values observed in control non-obese mice (Figure 6). Interestingly, caffeine increased the levels of these parameters, and they were significantly higher not only vs. levels in standard fed control group but also vs. obese control group.

Fig 6. Effect of administration of KD-64 or caffeine on TNF-α (A) and IL-6 (B) levels in plasma

Results are expressed as means ± SEM, n=8. Comparisons were performed by one-way ANOVA Dunnet's post hoc test. * Significant against control mice fed standard diet; ^ Significant against control mice fed fat/sugar diet; *,^ $p<0.05$, **,^^$p<0.01$.

3.6. Influence of KD-64 on AlaT activity in obese mice

Activity of AlaT in plasma of obese mice was significantly higher than in standard diet fed control mice. Administration of caffeine had no influence on AlaT activity, surprisingly KD-64 at the tested dose of 5 mg/kg significantly lowered AlaT levels in obese mice (Figure 7).

Fig 7. Effect of administration of KD-64 or caffeine on alanine aminotransferase activity in plasma

Results are expressed as means ± SEM, n=8. Comparisons were performed by one-way ANOVA Dunnet's post hoc test. * Significant against control mice fed standard diet, ^ Significant against control mice fed fat/sugar diet; *,^ $p<0.05$.

3.7. Glucose tolerance and insulin sensitivity after KD-64 treatment of obese mice

At 30 minutes after glucose load the blood glucose levels of mice in all tested groups receiving high fat diet were similar and significantly higher as compared to the levels

349 determined in control standard diet fed mice. At the subsequent time points (60 and 120
350 minutes after glucose load) there was no statistical difference observed between glucose blood
351 levels of standard diet fed mice and high fat diet fed mice treated with KD-64 or caffeine.
352 Interestingly, at the last time point (120 minutes after glucose load) glucose levels determined
353 only in mice treated with KD-64 compound were similar to the levels observed in control
354 standard diet fed mice and significantly lower than in control obese mice. Results are shown
355 in Figure 8A. At the same time, as shown in Figure 8B, the AUC was decreased by KD-64
356 treatment at the dose of 5 mg/kg b.w. as compared to both obese control group and group
357 treated with caffeine, however it was still higher than in control standard diet fed group.

358 **Fig 8. Glucose tolerance test**

359 (**A**) Intraperitoneal glucose tolerance test (IPGTT), (**B**) area under the curve of IPGTT.
360 Results are expressed as means ± SEM, n=8. Comparisons were performed by Multi-t test. *
361 Significant between control mice fed standard diet and control mice fed fat/sugar diet, ^
362 Significant between control mice fed standard diet and mice fed fat/sugar diet and treated with
363 KD-64, & Significant between control mice fed standard diet and mice fed fat/sugar diet and
364 treated with caffeine, $ Significant between control mice fed fat/sugar diet and mice fed
365 fat/sugar diet and treated with KD-64; $p<0.05, ***,^^^,&&&p<0.001.

366
367 In the insulin test, neither KD-64 nor caffeine affected blood glucose levels, which were
368 similar in all tested groups (Figure 9).

369 **Fig 9. Insulin sensitivity test**

370 (**A**) Insulin tolerance test (ITT), (**B**) area under the curve of the ITT. Results are expressed as
371 means ± SEM, n=8. Comparisons were performed by Multi-t test.

372
373 *3.8. Influence of KD-64 on spontaneous activity*

Compound KD-64 at the tested dose did not affect spontaneous activity in obese mice after a single *ip* administration, but spontaneous activity decreased during certain hours after twentieth administration vs. spontaneous activity in control group. Caffeine, on the other hand, increased spontaneous activity during certain hours after both first and twentieth *ip* administration of the tested dose. The results are shown in Figure 10.

Fig 10. Spontaneous activity after the first (A) and twentieth (B) administration of tested compounds

Results are expressed as means ± SEM, n=8. Comparisons were performed by Multi-t test. * Significant between control mice fed fat/sugar diet and mice fed fat/sugar diet and treated with KD-64, # Significant between control mice fed fat/sugar diet and mice fed fat/sugar diet and treated with caffeine; *#p<0.05, **,##p<0.01.

4. Discussion

The A_2 adenosine receptors play an important role in regulation of glucose homeostasis in both diabetes and obesity, but they also take active part in the inflammatory processes and these two particular abilities of A_2 adenosine receptors were a subject of the presented study. In the obesity model we have tested activity of the new selective antagonist of A_{2A} adenosine receptor – compound KD-64 and compared its effect to the known non-selective antagonist of adenosine receptors – caffeine. Subsequently in two different inflammation models the activity of KD-64 has been compared to the activity of potent anti-inflammatory agent – ketoprofen.

From the available literature it is known that caffeine – non-selective adenosine A_{2A} receptor antagonist is able to inhibit various obesity-related abnormalities, including low metabolism, adiposity, dyslipidemia, systemic/tissue inflammation, and insulin resistance [51]. Thus, we

398 began to wonder whether the selective adenosine A_{2A} receptor antagonist may have similar
399 properties – especially when it comes to inflammation and obesity?
400 For research, the selective A_{2A} adenosine receptor antagonist KD-64 has been chosen, which
401 in previous studies, administered at the dose of 5 mg/kg b.w., exerted antiparkinsonian
402 activity [52]. Preliminary experiments aimed at the determination of spontaneous and anti-
403 inflammatory activities after a single administration of the tested compound. The purpose of
404 this study was to select the lowest dose of KD-64 compound that has anti-inflammatory
405 activity, but simultaneously does not influence spontaneous activity so it can be administered
406 chronically in the obesity model. In obesity studies it is particularly important that the tested
407 compounds do not increase activity, which could further contribute to an increase in energy
408 consumption and an undesirable effect of the psyche - agitation. On the other hand, it is also
409 important that the tested compounds do not reduce activity, because sedation, for example,
410 may cause a decrease in food intake and consequently weight loss that could be attributed as a
411 non-specific effect. Such effect will also be unacceptable in chronic therapy since chronic
412 fasting can be harmful to the body. Therefore, for the safe and effective compounds tested in
413 obesity models and with potential action towards reducing body weight, it is crucial to have
414 no effect on spontaneous activity [53]. In the case of presented studies it is especially
415 important since adenosine, through A_1 and A_{2A} receptors, is involved in the regulation of
416 spontaneous activity [54, 55]. It has been reported that adenosine A_{2A} agonists, e.g. the CGS
417 21680 compound, can cause sedation [56], while antagonists of this receptor, e.g. caffeine,
418 have a stimulating effect [57].
419 Our research showed that the selective A_{2A} receptor antagonist KD-64 did not induce changes
420 in spontaneous activity after a single administration therefore we proceeded with the further
421 experiments. In models of both local and systemic inflammation the anti-inflammatory effect
422 of the KD-64 compound administered at a dose of 5 mg/kg b.w./day was comparable to the

anti-inflammatory effect of ketoprofen (5 mg/kg b.w./day) used as a reference standard. There are reports in the literature that pharmacological blockade of selected adenosine receptor subtypes (PSB-36, PSB-1115, MSX-3, and PSB-10) after systemic application of antagonists generally leads to decrease in edema formation after the carrageenan injection [58]. In the case of A_{2A} antagonist, activity of the drug varies with time, suggesting that the importance of adenosine receptor activation in the inflammatory process dynamically changes in the course of inflammation [58]. The local injection of the highly A_{2A}-selective agonist CGS21680 induced paw edema, conversely [59] A_{2A} antagonist MSX-3, at a dose of 10 mg/kg b.w., significantly reduced the carrageenan-induced edema [58]. Similarly, in our study, we showed that intraperitoneal injection of selective A_{2A} receptor antagonist KD-64 at a dose of 5 mg/kg b.w./day, results in a potent inhibition of carrageenan-induced edema formation (Figure 3). Thus, the results obtained are in line with literature reports and clearly show that selective A_{2A} receptor antagonists have anti-inflammatory effect.

Adenosine may be added to the growing list of key signalling molecules that regulate vascular function and homeostasis and to a very selected list of agonists that promote integrity of the vascular bed [60]. There are reports in the literature that adenosine regulates the pulmonary endothelial cells barrier function via A_{2A} receptors [60] and through its influence on the adenosine receptors A_1 and A_{2A} can modulate for example blood-brain barrier permeability [61]. Blocking just the adenosine A_{2A} receptor reduces permeability and blocks the entry of inflammatory cells and soluble factors into the brain [61]. 1It has been also reported that vascular permeability in the hind plantar skin of rats decreases following lumbar sympathectomy, possibly via reduction of adenosine receptor A_{2A} expression [62]. In the second model of inflammation used in the presented study i.e. zymosan-induced peritonitis, KD-64 showed significant anti-inflammatory effect, manifested by decrease of both vascular permeability, and plasma neutrophils count. The results were comparable to the ones obtained

448 after ketoprofen administration. It is an important finding, confirming that A_{2A} adenosine
449 receptor blockade may be directly responsible for a decrease in vascular permeability.
450 Anti-inflammatory effect of KD-64 was also evaluated through the measurement of CRP
451 levels - an acute phase protein primarily expressed and secreted by the liver. In response to
452 tissue injury or infection, the plasma concentrations of CRP can increase rapidly, moreover
453 CRP level also increases in chronic inflammatory diseases, including cardiovascular and
454 autoimmune disease. Due to the correlation between CRP and inflammation, CRP has
455 attracted wide attention as a non-specific marker used for purpose of evaluation and
456 monitoring of the infection and inflammation development as well as a prognostic marker for
457 cardiovascular events [63]. In both models of inflammation, the tested antagonist of A_{2A}
458 adenosine receptors KD-64 statistically significantly reduced the level of CRP in plasma. This
459 confirms the anti-inflammatory efficacy of this compound after its *ip* administration at a dose
460 of 5 mg/kg b.w./day.

461 Based on the described above findings the dose of 5 mg/kg b.w./day of KD-64 was selected
462 for testing in mice obesity model. During the first days of KD-64 administration, obese
463 animals weighed significantly less compared to the obese control mice. But then they began
464 to gain weight at the rate comparable to obese control mice. Not surprisingly, the amount of
465 peritoneal adipose tissue, measured at the end of the experiment, was comparable in these two
466 groups of animals. It is interesting, however, that the group receiving caffeine at a dose of 50
467 mg/kg/ b.w./day which gained weight significantly less than obese control mice (results
468 consistent with the literature findings) [64] at the end of the experiment had the amount of
469 peritoneal fat also comparable to the other experimental groups.
470 As the compound KD-64 was administered, its effectiveness in reducing weight of obese mice
471 was decreasing. Unfortunately, the conducted research does not provide an assessment of why
472 this might have happened. More detailed investigation is needed to determine if, for example,

it was due to changes in the number and sensitivity of adenosine receptors with repeated administrations. Literature reports such cases that repeated administrations of adenosine A_{2A} ligands may lead to the changes in regulation (both up- or down-regulation) of the A_{2A} adenosine receptor gene [65]. In addition, various pathological conditions can cause changes in the density of A_{2A} adenosine receptors, for example it has been shown that pro-inflammatory stimuli up-regulate A_{2A} adenoside receptor and for the effective treatment appropriately higher doses of ligands are required [66]. A reduction in DNA methylation at the A_{2A} adenosine receptor gene promoter site and an increase in the protein levels and gene expression in binge-like-eating rats has been also reported [65]. Interestingly, alterations of DNA methylation of A_{2A} adenosine receptor has been observed in other diseases such as schizophrenia (67), Huntington's disease [68] and cardiomyopathies [69].

Studies of spontaneous activity in obese mice showed that repeated administration of KD-64 led to a decrease in spontaneous activity at some hours after the twentieth administration of this compound. As previously mentioned, adenosine A_{2A} agonists, e.g. the CGS 21680 compound, can cause sedation [56], which may indicate that in fact a change in receptor density and sensitivity (up-regulation) after repeated administration of KD-64 could be the cause of such observation.

In obesity, the white adipose tissue produces large numbers of inflammatory agents including TNF-α and IL-6, which can affect the physiology of the adipose tissue locally, but also may induce systemic effects on the other organs [70]. IL-6 has emerged as one of the mediators linking obesity-derived chronic inflammation with insulin resistance. In high fat diet fed obese mice, activated hepatic IL-6 signalling is accompanied by systemic and local insulin resistance, which can be reversed by neutralization of IL-6 [71]. Indeed, in our study, the levels of TNF-α and IL-6 in the plasma of obese mice were higher as compared to control non-obese mice and were significantly reduced by the administration of KD-64. This

498 reduction in the levels of inflammatory cytokines is probably the result of the anti-
499 inflammatory effect of the tested compound. It should be emphasized that these tests were
500 made with homogeneous, very sensitive and reliable methods. In addition, in the glucose load
501 test, significant differences were observed in the response curve in group which received KD-
502 64 treatment compared to the obese control group. The glucose level in mice treated with KD-
503 64 one hour after loading did not differ statistically from the level determined in non-obese
504 mice fed standard feed. This indicates an improvement in glucose tolerance in KD-64-treated
505 mice compared to the obese control mice which might be connected to the anti-inflammatory
506 effect of the tested compound and a decrease in plasma IL-6 levels, since IL-6 and its
507 signalling path play complex roles in metabolic disorders [72]. It was recently reported that
508 IL-6 enhances fatty acid synthesis in murine hepatocytes via the induction of the citrate
509 transporter *mIndy* [73] and it also exacerbates hepatic inflammation and steatosis [74].
510 Additionally, adenosine has been shown to promote IL-6 production [75] and our study
511 demonstrates that adenosine A_{2A} receptor may be associated with this activity, because
512 selective antagonist of this particular receptor decreased IL-6 level in plasma. In contrast,
513 several human studies reported that despite the well-proven anti-inflammatory effect of
514 caffeine [77, 78, 79], its administration, leads to an increase in serum IL-6 levels [79, 80]. The
515 results of our study, even though performed on mice, are in line with these observations.
516 Observed in our studies differences in plasma IL-6 levels between the caffeine and KD-64
517 treatment groups may indicate that decrease in IL-6 concentration is due not only to the anti-
518 inflammatory effect of the KD-64 compound, but also adenosine A_{2A} receptor blockade. This
519 is an interesting topic that undoubtedly requires further research.
520 Another interesting and very favourable result of KD-64 treatment is its ability to normalize
521 the elevated AlaT activity, induced by high-fat/sugar feeding. Liver is a vital organ involved
522 in detoxification and drug metabolism, therefore potential hepatotoxicity could eliminate the

test compound from further stages of development especially if it is intended for longer use. In our experiment caffeine did not have any effect on AlaT levels, although there are reports in the literature that in the obesity model caused by the administration of high-fat feed, caffeine administered for several weeks at the dose of 20-40 mg/kg in the drinking water normalizes liver enzymes [65]. Probably this effect depends also on the dose and time of caffeine administration.

In conclusion, after repeated administrations of a selective A_{2A} adenosine receptor antagonist compound KD-64 with documented anti-inflammatory activity, no significant reduction in weight gain was observed in obese mice fed high-calorie feed. However, contrary to caffeine (non-selective adenosine receptor antagonist) investigated compound normalized levels of selected cytokines and inflammatory proteins, including IL-6, as well as, activity of alanine transaminase and improved glucose tolerance in the obese mice. Thus our findings prove that the activity of the selective adenosine A_{2A} receptor antagonist is different from that of the non-selective antagonist.

5. Acknowledgments

The authors wish to gratefully acknowledge Maria Kaleta and Joanna Knutelska for their technical assistance.

6. Conflict of Interest: The authors declare that they have no conflict of interest.

7. References

1. Shoelson SE, Herrero L, Naaz A. Obesity, inflammation and insulin resistance. Gastroenterology. 2007;132:2169-2180.

2. Saltiel AR, Olefsky JM. Inflammatory mechanisms linking obesity and metabolic disease. J Clin Invest. 2017;127:1-4.

3. Lee EB, Mattson MP. The neuropathology of obesity: Insights from human disease. Acta Neuropathologica. 2014;127:3-28.

548 4. Trayhurn P. Adipose tissue in obesity - An inflammatory issue. Endocrinology.
549 2005;146:1003-1005.
550 5. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic
551 disease. Nature Reviews Immunology. 2011;11,85–97.
552 6. Fernández-Sánchez A, Madrigal-Santillán E, Bautista M, Esquivel-Soto J, Morales-
553 González A, Esquivel-Chirino C, et al. Inflammation, oxidative stress, and obesity. Int J Mol
554 Sci. 2011;12:3117-3132.
555 7. Ellulu MS, Patimah I, Khaza'ai H, Rahmat A, Abed Y. Obesity and inflammation: The
556 linking mechanism and the complications. Arch Med Sci. 2017;13(4):851-863.
557 8. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa JI, et al. Paradoxical
558 decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res
559 Commun. 1999;57:79-83.
560 9. Azizian M, Mahdipour E, Mirhafez SR, Shoeibi S, Nematy M, Esmaily H, et al. Cytokine
561 profiles in overweight and obese subjects and normal weight individuals matched for age and
562 gender. Ann Clin Biochem. 2016;53:663-668.
563 10. Leon-Cabrera S, Arana-Lechuga Y, Esqueda-León E, Terán-Pérez G, Gonzalez-Chavez
564 A, Escobedo G. Reduced Systemic levels of IL-10 are associated with the severity of
565 obstructive sleep apnea and insulin resistance in morbidly obese humans. Mediators Inflamm.
566 2015;2015:493409. doi: 10.1155/2015/493409
567 11. Trayhurn P, Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose
568 tissue. Br J Nutr. 2004;92:347-355.
569 12. Gregor MF, Hotamisligil GS. Thematic review series: Adipocyte Biology. Adipocyte
570 stress: the endoplasmic reticulum and metabolic disease. J Lipid Res. 2007;48:1905–1914.
571 13. Nathan C. Epidemic inflammation: pondering obesity. Moi Med. 2008;14:485-492.
572 14. Giri B, Dey S, Das T, Sarkar M, Banerjee J, Dash SK. Chronic hyperglycemia mediated

physiological alteration and metabolic distortion leads to organ dysfunction, infection, cancer progression and other pathophysiological consequences: An update on glucose toxicity. Biomed and Pharmac. 2018;107:306-328

15. Cully M. Obesity: Adenosine protects from diet-induced obesity. Nat Rev Drug Discov. 2014;13:886–887.

16. Haskó G, Linden J, Cronstein B, Pacher P. Adenosine receptors: Therapeutic aspects for inflammatory and immune diseases. Nature Reviews Drug Discovery. 2008;7:759-770.

17. Pardo F, Villalobos-Labra R, Chiarello DI, Salsoso R, Toledo F, Gutierrez J, et al. Molecular implication of adenosine in obesity. Mol Aspect Medicine. 2017;55: 90-101.

18. Haskö G, Pascher P. A2A receptors in inflammation and injury: lessons learned from transgenic animals. J Leukoc Biol. 2008;83:447–455.

19. Gnad T, Scheibler S, Kugelgen, Scheele C, Ana Kilic, Glo A, et al. Adenosine activates brown adipose tissue and recruits beige adipocytes via A_{2A} receptors. Nature. 2014;516:395-399.

20. Yaar R, Jones MR, Chen JF, Ravid K. Animal models for the study of adenosine receptor function. J Cell Physiol. 2005;202:9-20.

21. Csóka B, Törő G, Vindeirinho J, Varga ZV, Koscsó B, Németh ZH, et al. A_{2A} adenosine receptors control pancreatic dysfunction in high-fat-diet-induced obesity. FASEB J. 2017;31:4985-4997.

22. Fink JS, Weaver DR, Rivkees SA, Peterfreund RA, Pollack AE, Adler EM. Molecular cloning of the rat A2 adenosine receptor: selective co-expression with D_2 dopamine receptors in rat striatum. Mol Brain Res. 1992;14:185-195.

23. Fenu S, Pinna A, Ongini E, Morelli M. Adenosine A2A receptor antagonism potentiates L-DOPA-induced turning behaviour and c-fos expression in 6-hydroxydopamine-lesioned rats. Eur J Pharmacol. 1997;321:143-147.

598 24. Cincotta AH, Tozzo E, Scislowski PW. Bromocriptine/SKF38393 treatment ameliorates
599 obesity and associated metabolic dysfunctions in obese (ob/ob) mice. Life Sci. 1997;61:951-
600 956.
601 25. Scislowski PWD, Tozzo E, Zhang Y, Phaneuf S, Prevelige R, Cincotta AH. Biochemical
602 mechanisms responsible for the attenuation of diabetic and obese conditions in ob/ob mice
603 treated with dopaminergic agonists. Int J Obes. 1999;23:425-431.
604 26. Bina KG, Cincotta A. Dopaminergic agonists normalize elevated hypothalamic
605 neuropeptide Y and corticotropin-releasing hormone, body weight gain, and hyperglycemia in
606 ob/ob mice. Neuroendocrinology. 2000;71:68-78.
607 27. Davis CA, Levitan RD, Reid C, Carter JC, Kaplan AS, Patte KA, et al. Dopamine for
608 "waiting" and opioids for "liking" a comparison of obese adults with and without binge
609 eating. Obes (Silver Spring). 2009;17:1220-1225.
610 28. Reece TB, Ellman PI, Maxey TS, Crosby IK, Warren PS, Chong TW, et al. Adenosine
611 A_{2A} receptor activation reduces inflammation and preserves pulmonary function in an in vivo
612 model of lung transplantation. J Thorac Cardiovasc Surg. 2005;129:1137-1143.
613 29. Sharma AK, Linden J, Kron IL, Laubach VE. Protection from pulmonary ischemia-
614 reperfusion injury by adenosine A_{2A} receptor activation. Respir Res. 2009 Jun 26;10:58. doi:
615 10.1186/1465-9921-10-58.
616 30. Li L, Hao JX, Fredholm BB, Schulte G, Wiesenfeld-Hallin Z, Xu XJ. Peripheral
617 adenosine A_{2A} receptors are involved in carrageenan-induced mechanical hyperalgesia in
618 mice. Neuroscience. 2010;170:923-928.
619 31. Zygmunt M, Gołembiowska K, Drabczyńska A, Kieć-Kononowicz K, Sapa J. Anti-
620 inflammatory, antioxidant, and antiparkinsonian effects of adenosine A_{2A} receptor antagonists.
621 Pharmacol Biochem Behav. 2015;132:71-78.

622 32. Carrageta DF, Dias TR, Alves MG, Oliveira PF, Monteiro MP, Silva BM. Anti-obesity
623 potential of natural methylxanthines. J of Func Foods. 2018;43:84–94.
624 33. Dulloo AG. The search for compounds that stimulate thermogenesis in obesity
625 management: From pharmaceuticals to functional food ingredients. Obes Rev. 2011;12:866-
626 883.
627 34. Lopes-Silva JP, Ferreira Da Silva Santos J, Branco BHM, Abad CCC, Farias De Oliveira
628 L, Loturco I. Caffeine ingestion increases estimated glycolytic metabolism during taekwondo
629 combat simulation but does not improve performance or parasympathetic reactivation. PLoS
630 One. 2015;5:1-16.
631 35. Bakuradze T, Parra GAM, Riedel A, Somoza V, Lang R, Dieminger N, et al. Four-week
632 coffee consumption affects energy intake, satiety regulation, body fat, and protects DNA
633 integrity. Food Res Int. 2014;63:420–427.
634 36. Lopez-Garcia E, Van Dam RM, Rajpathak S, Willett WC, Manson JAE, Hu FB. Changes
635 in caffeine intake and long-term weight change in men and women. Am J Clin Nutr.
636 2006;83:674-680.
637 37. Liu AG, Smith SR, Fujioka K, Greenway FL. The effect of leptin, caffeine/ephedrine, and
638 their combination upon visceral fat mass and weight loss. Obesity. 2013;314:433–447.
639 38. Liu AG, Arceneaux KP, Chu JT, Jacob G, Schreiber AL, Tipton RC, et al. The effect of
640 caffeine and albuterol on body composition and metabolic rate. Obesity. 2015;23:1830-1835.
641 39. Davoodi SH, Hajimiresmaiel SJ, Ajami M, Mohseni-Bandpei A, Ayatollahi SA,
642 Dowlatshahi K, et al. Caffeine treatment prevented from weight regain after calorie shifting
643 diet induced weight loss. Iran J Pharm Res. 2014;13:707–718.
644 40. Schubert MM, Hall S, Leveritt M, Grant G, Sabapathy S, Desbrow B. Caffeine
645 consumption around an exercise bout: Effects on energy expenditure, energy intake, and
646 exercise enjoyment. J Appl Physiol. 2014;117:745-754.

647 41. Drabczyńska A, Yuzlenko O, Köse M, Paskaleva M, Schiedel AC, Karolak-
648 Wojciechowska J, et al. Synthesis and biological activity of tricyclic cycloalkylimidazo-,
649 pyrimido- and diazepinopurinediones. Eur J Med Chem. 2011;46:3590-3607.
650 42. Drabczyńska A, Zygmunt M, Sapa J, Filipek B, Müller CE, Kieć-Kononowicz K.
651 Antiparkinsonian effects of novel adenosine A_{2A} receptor antagonists. Arch Pharm
652 (Weinheim). 2011;344:20-27.
653 43. Winter CA, Risley EA, Nuss GW. Carrageenin-Induced Edema in Hind Paw of the Rat as
654 an Assay for Antiinflammatory Drugs. Proc Soc Exp Biol Med. 1962;111:544-547.
655 44. Yazawa K, Suga K, Honma A, Shirosaki M, Koyama T. Anti-inflammatory effects of
656 seeds of the tropical fruit camu-camu (Myrciaria dubia). J Nutr Sci Vitaminol (Tokyo).
657 2011;57:104-107.
658 45. Kwiecień B, Dudek B, Bilska-Wilkosz A, Knutelska J, Bednarski M, Kwiecień I, et al. In
659 vivo Anti-inflammatory Activity of Lipoic Acid Derivatives in Mice. Postepy Hig Med Dosw.
660 2013;67:331-338.
661 46. Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, et al.
662 Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem
663 Biophys Res Commun. 1999;57:79-83.
664 47. Kołaczkowska E, Barteczko M, Plytycz B, Arnold B. Role of lymphocytes in the course
665 of murine zymosan-induced peritonitis. Inflamm Res. 2008;57:272-278.
666 48. Dudek M, Kuder K, Kołaczkowski M, Olczyk A, Żmudzka E, Rak A, et al. H3 histamine
667 receptor antagonist pitolisant reverses some subchronic disturbances induced by olanzapine in
668 mice. Metab Brain Dis. 2016;31:1023-1029.
669 49. Kotańska M, Kuder KJ, Szczepańska K, Sapa J, Kieć-Kononowicz K. The histamine H3
670 receptor inverse agonist pitolisant reduces body weight in obese mice. Naunyn
671 Schmiedebergs Arch Pharmacol. 2018;391:875-881.

672 50. Kotańska M, Mika K, Reguła K, Szczepańska K, Szafarz M, Bednarski M, et al. KSK19 -
673 Novel histamine H3 receptor ligand reduces body weight in diet induced obese mice.
674 Biochem Pharmacol. 2019;168:193-203.
675 51. Liu CW, Tsai HC, Huang CC, Tsai CY, Su YB, Lin MW, et al. Effects and mechanisms
676 of caffeine to improve immunological and metabolic abnormalities in diet-induced obese rats.
677 Am J Physiol Endocrinol Metab. 2018;314(5):433-447.
678 52. Drabczyńska A, Zygmunt M, Sapa J, Filipek B, Müller CE, Kieć-Kononowicz K.
679 Antiparkinsonian effects of novel adenosine A(2A) receptor antagonists. Arch Pharm
680 (Weinheim). 2011;344:20-27.
681 53. Dudek M, Marcinkowska M, Bucki A, Olczyk A, Kołaczkowski M. Idalopirdine – a small
682 molecule antagonist of 5-HT6 with therapeutic potential against obesity. Metab Brain Dis.
683 2015;30:1487-1494.
684 54. Yacoubi M El, Ledent C, Ménard J-F, Parmentier M, Costentin J, Vaugeois JM. The
685 stimulant effects of caffeine on locomotor behaviour in mice are mediated through its
686 blockade of adenosine A2A receptors. Br J Pharmacol. 2000;129:1465-1473.
687 55. Stenberg D. Neuroanatomy and neurochemistry of sleep. Cell Mol Life Sci 2007;64:1187-
688 1204
689 56. Mingote S, Pereira M, Farrar AM, McLaughlin PJ, Salamone JD. Systemic administration
690 of the adenosine A2A agonist CGS 21680 induces sedation at doses that suppress lever
691 pressing and food intake. Pharmacol Biochem Behav. 2008;89:345-351.
692 57. Bădescu SV, Tătaru CP, Kobylinska L, Georgescu EL, Zahiu DM, Zăgrean AM, et al.
693 Effects of caffeine on locomotor activity in streptozotocin-induced diabetic rats. J Med Life.
694 2016;9:275-279.
695 58. Bilkei-Gorzo A, Abo-Salem OM, Hayallah AM, Michel K, Müller CE, Zimmer A.
696 Adenosine receptor subtype-selective antagonists in inflammation and hyperalgesia. Naunyn

Schmiedebergs Arch Pharmacol. 2008;377:65-67.

59. Sawynok J, Reid A, Liu XJ. Acute paw oedema induced by local injection of adenosine A1, A2 and A3 receptor agonists. Eur J Pharmacol. 1999;386:253-261.

60. Umapathy NS, Fan Z, Zemskov EA, Alieva IB, Black SM, Verin AD. Molecular mechanisms involved in adenosine-induced endothelial cell barrier enhancement. Vascul Pharmacol. 2010;52:199-206.

61. Bynoe MS, Viret C, Yan A, Kim DG. Adenosine receptor signaling: A key to opening the blood-brain door. Fluids and Barriers of the CNS. Fluids Barriers CNS. 2015;2:12-20.

62. Zheng Z, Liu Y, Zhu L, Tang J, Huang W, Cheng B. Lumbar sympathectomy reduces vascular permeability, possibly through decreased adenosine receptor A2a expression in the hind plantar skin of rats. Clin Hemorheol Microcirc. 2018;68:5-15.

63. Yao ZY, Zhang Y, Wu HB. Regulation of C-reactive protein conformation in inflammation. Inflam Res. 2019;68:815-823.

64. Fang C, Cai X, Hayashi S, Hao S, Sakiyama H, Wang X, et al. Caffeine-stimulated muscle IL-6 mediates alleviation of non-alcoholic fatty liver disease. Biochim Biophys Acta - Mol Cell Biol Lipids. 2019;1864:271-280.

65. Micioni Di Bonaventura MV, Pucci M, Giusepponi ME, Romano A, Lambertucci C, Volpini R, et al. Regulation of adenosine A_{2A} receptor gene expression in a model of binge eating in the amygdaloid complex of female rats. J Psychopharmacol. 2019;33:1550-1561

66. Alfaro TM, Rodrigues DI, Tomé ÂR, Cunha RA, Robalo Cordeiro C. Adenosine A_{2A} receptors are up-regulated and control the activation of human alveolar macrophages. Pulm Pharmacol Ther. 2017;45:90-94.

67. Villar-Menéndez I, Díaz-Sánchez S, Blanch M, Albasanz JL, Pereira-Veiga T, Monje A. Reduced striatal adenosine A_{2A} receptor levels define a molecular subgroup in schizophrenia. J Psychiatr Res. 2014;51:49-59.

722 68. Villar-Menéndez I, Blanch M, Tyebji S, Pereira-Veiga T, Albasanz JL, Martín M.
723 Increased 5-methylcytosine and decreased 5-hydroxymethylcytosine levels are associated
724 with reduced striatal A2AR levels in Huntington's disease. NeuroMolecular Med.
725 2013;15:295-309.
726 69. Haas J, Frese KS, Park YJ, Keller A, Vogel B, Lindroth AM. Alterations in cardiac DNA
727 methylation in human dilated cardiomyopathy. EMBO Mol Med. 2013;5:413-429.
728 70. Ye J. Mechanisms of insulin resistance in obesity. Frontiers of Medicine in China.
729 2013;7:14-24.
730 71. Liu CW, Tsai HC, Huang CC, Tsai CY, Su YB, Lin MW, et al. Effects and mechanisms
731 of caffeine to improve immunological and metabolic abnormalities in diet-induced obese rats.
732 Am J Physiol Endocrinol Metab. 2018;314(5):433-447.
733 72. Ishihara K, Hirano T. IL-6 in autoimmune disease and chronic inflammatory proliferative
734 disease. Cytokine and Growth Factor Rev. 2002;13:357-368.
735 73. von Loeffelholz C, Lieske S, Neuschäfer-Rube F, Willmes DM, Raschzok N, Sauer IM, et
736 al. The human longevity gene homolog INDY and interleukin-6 interact in hepatic lipid
737 metabolism. Hepatology. 2017;66:616-630.
738 74. Hong F, Radaeva S, Pan HN, Tian Z, Veech R, Gao B. Interleukin 6 alleviates hepatic
739 steatosis and ischemia/reperfusion injury in mice with fatty liver disease. Hepatology.
740 2004;40:933-941.
741 75. Sitaraman SV, Merlin D, Wang L, Wong M, Gewirtz AT, Si-Tahar M. Neutrophil-
742 epithelial crosstalk at the intestinal lumenal surface mediated by reciprocal secretion of
743 adenosine and IL-6. J Clin Invest. 2001;107:861-869.
744 76. Chavez-Valdez R, Wills-Karp M, Ahlawat R, Cristofalo EA, Nathan A, Gauda EB.
745 Caffeine modulates TNF-α production by cord blood monocytes: The role of adenosine
746 receptors. Pediatr Res. 2009;65:203-308.

747 77. Lv X, Chen Z, Li J, Zhang L, Liu H, Huang C, et al. Caffeine protects against alcoholic
748 liver injury by attenuating inflammatory response and oxidative stress. Inflamm Res.
749 2010;59:635-645.
750 78. Iris M, Tsou PS, Sawalha AH. Caffeine inhibits STAT1 signaling and downregulates
751 inflammatory pathways involved in autoimmunity. Clin Immunol. 2018;192:68-77.
752 79. Walker GJ, Finlay O, Griffiths H, Sylvester J, Williams M, Bishop NC. Immunoendocrine
753 response to cycling following ingestion of caffeine and carbohydrate. Med Sci Sports Exerc.
754 2007;39:1554-1560.
755 80. Tauler P, Martinez S, Moreno S, Monjo M, Martínez P, Aguiló A. Effects of caffeine on
756 the inflammatory response induced by a 15-km run competition. Med Sci Sports Exerc.
757 2013;45:1269–1276.

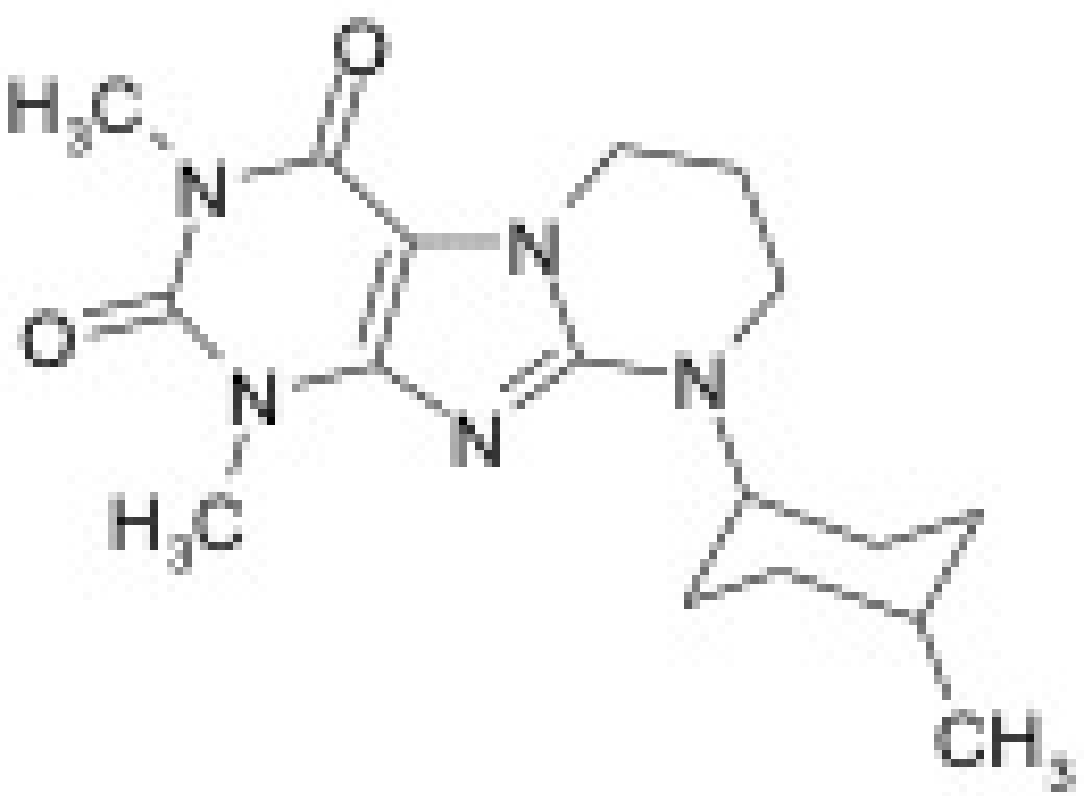


Figure 1

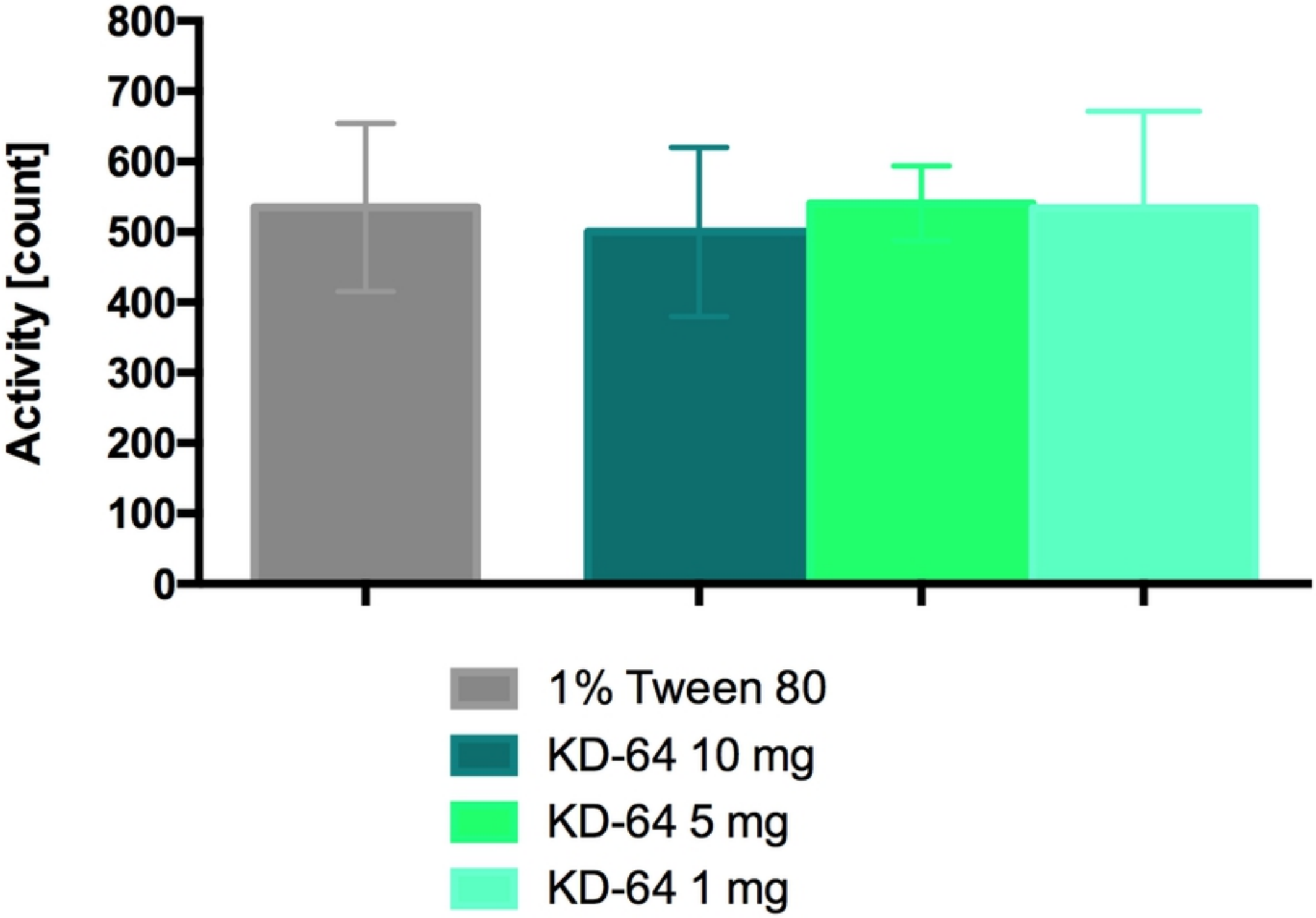
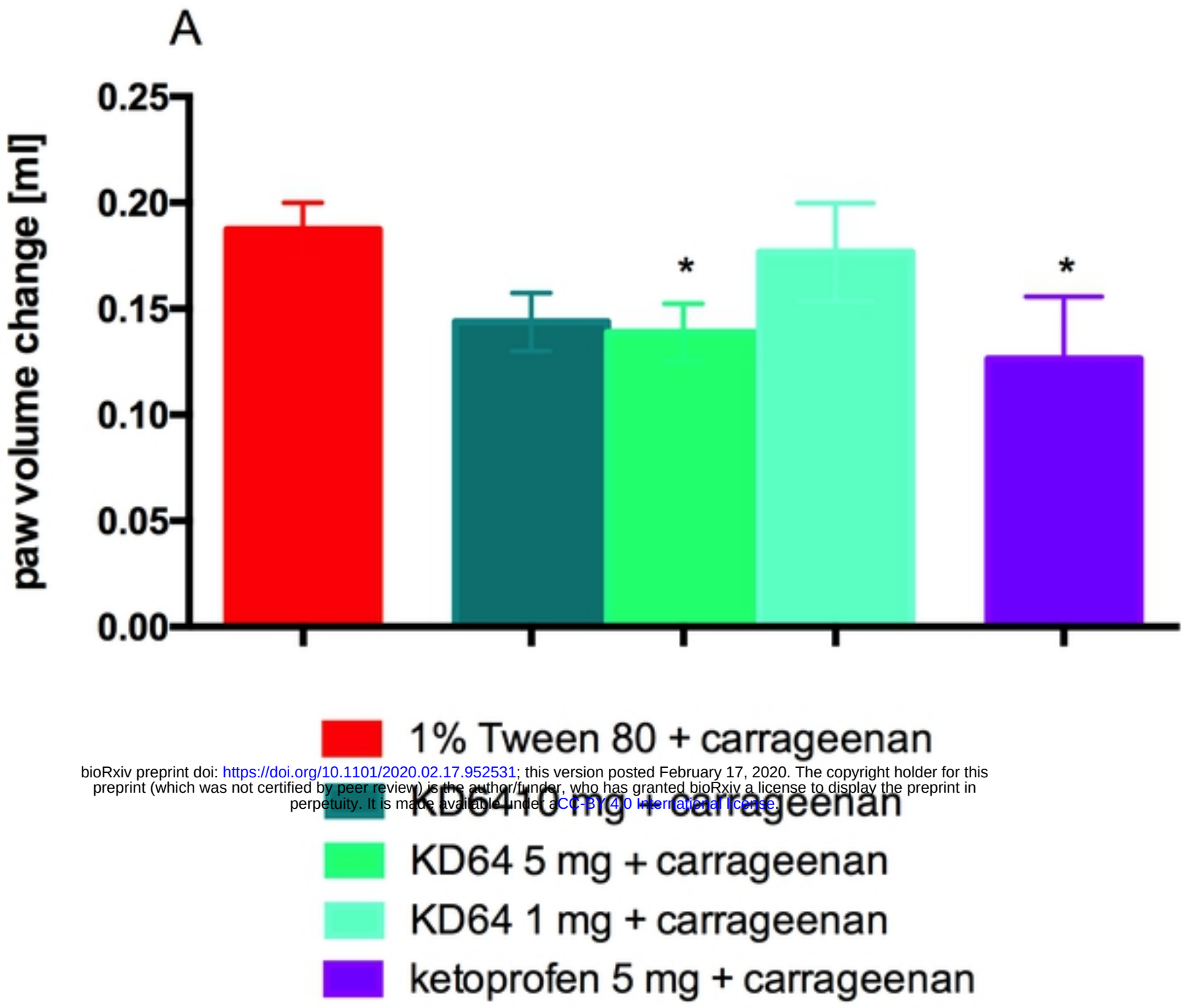

800
700
600
500
400
300
200
100
0
Activity [count]
1% Tween 80
KD-64 10 mg
KD-64 5 mg
KD-64 1 mg

Figure 2



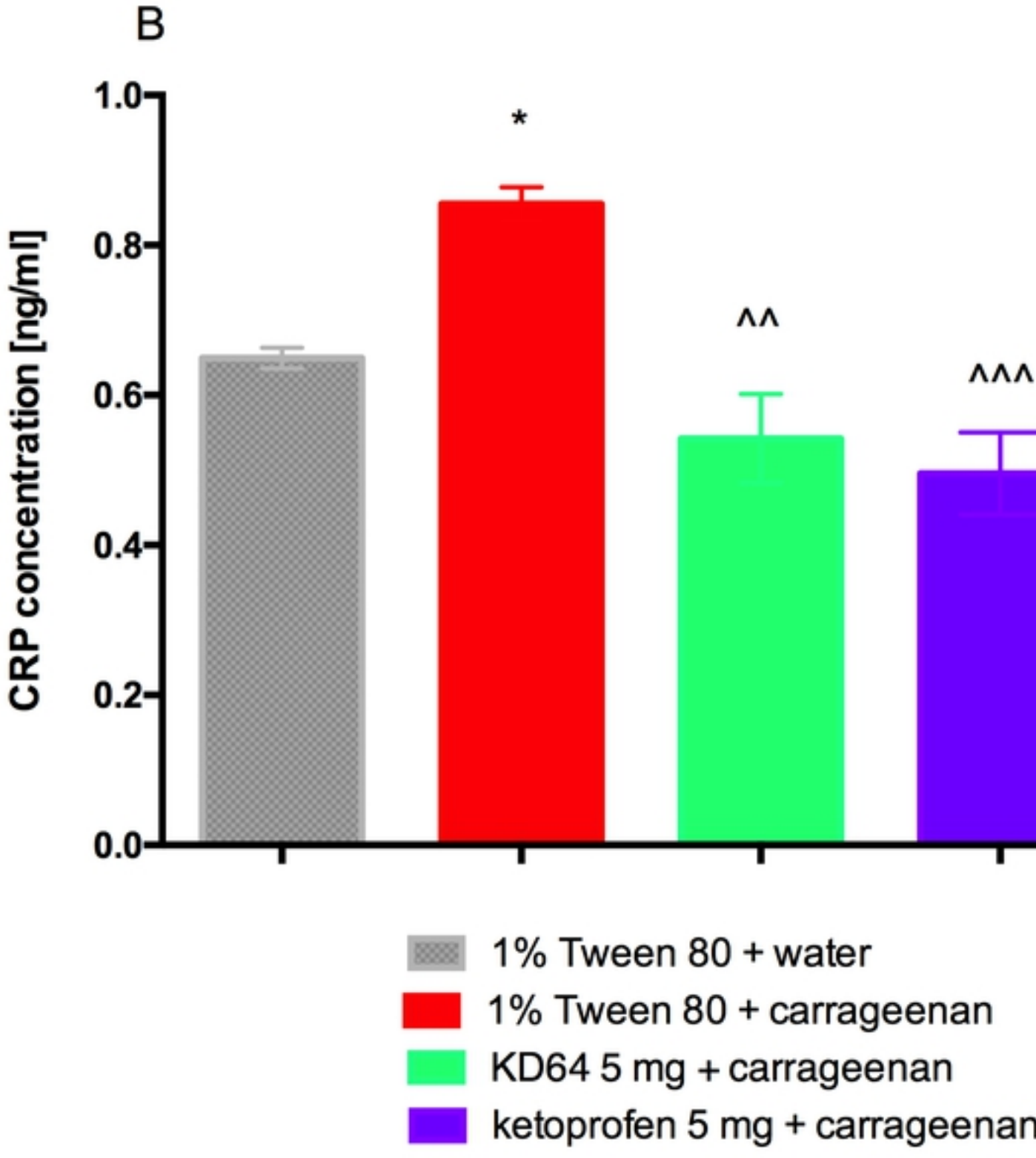
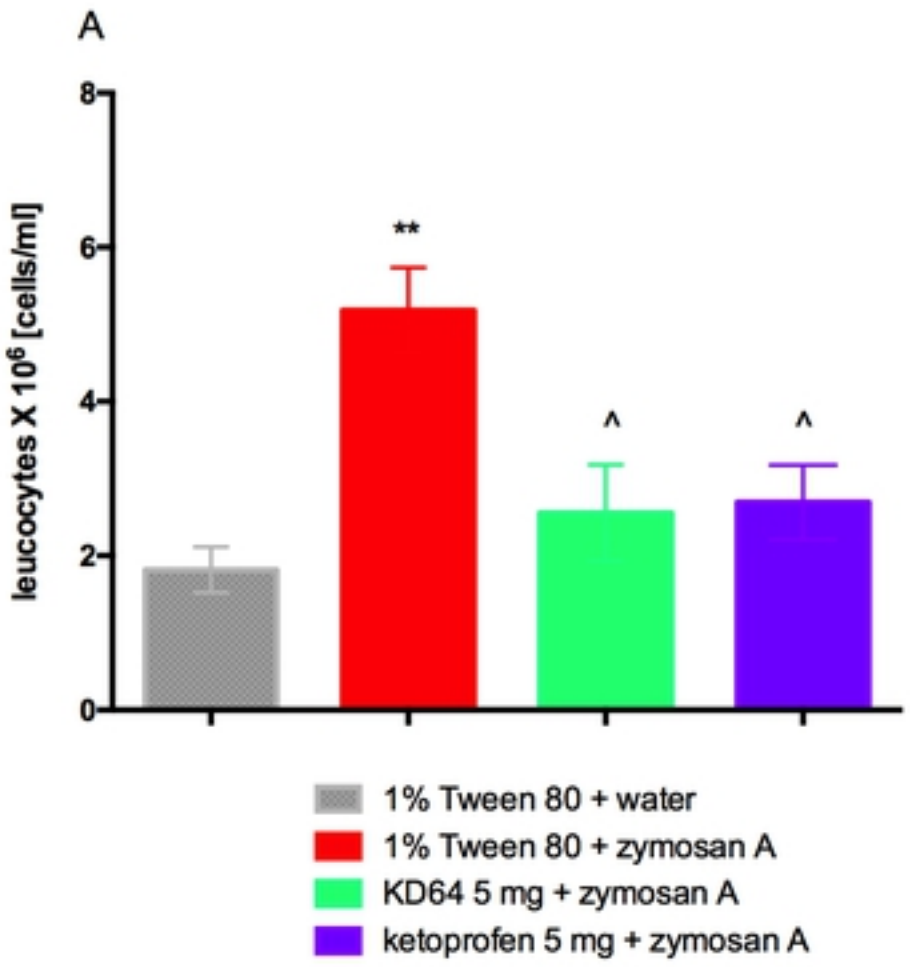
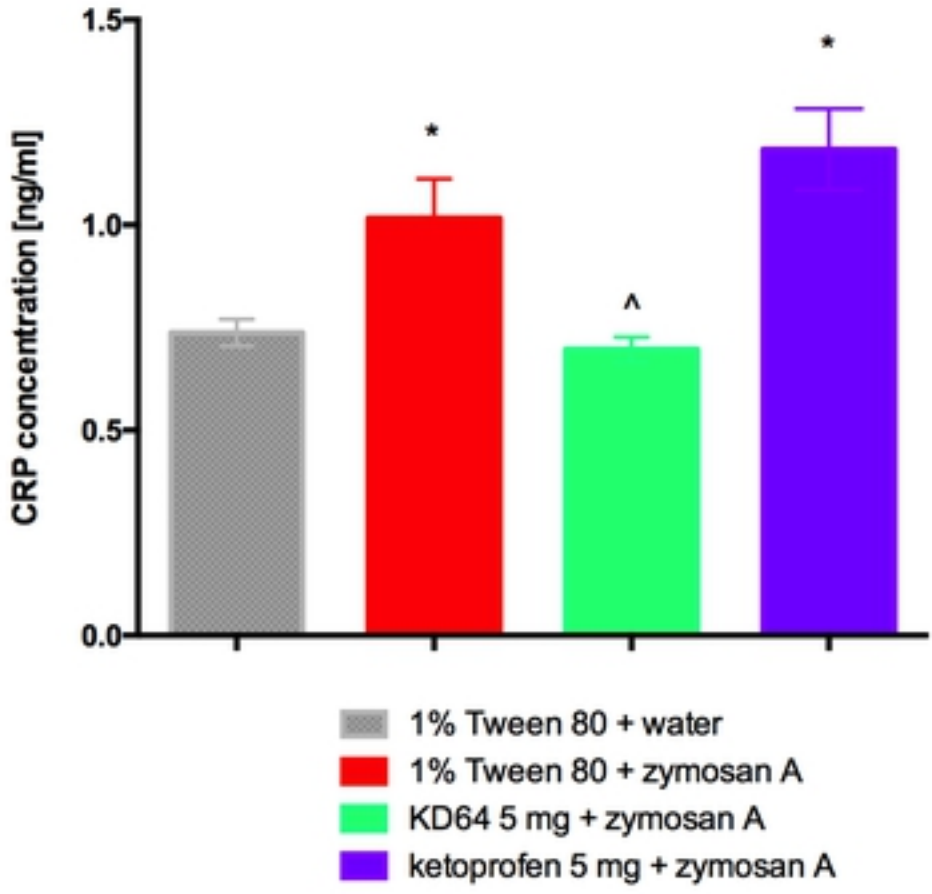


Figure 3





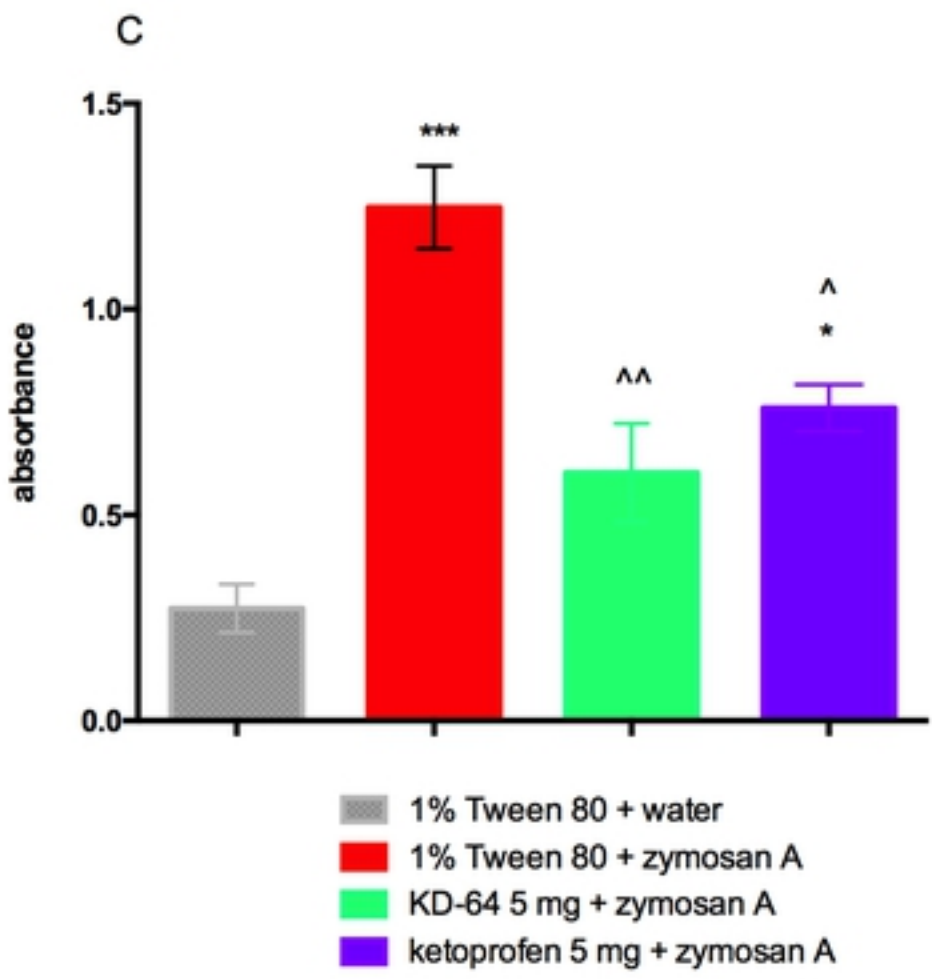


Figure 4

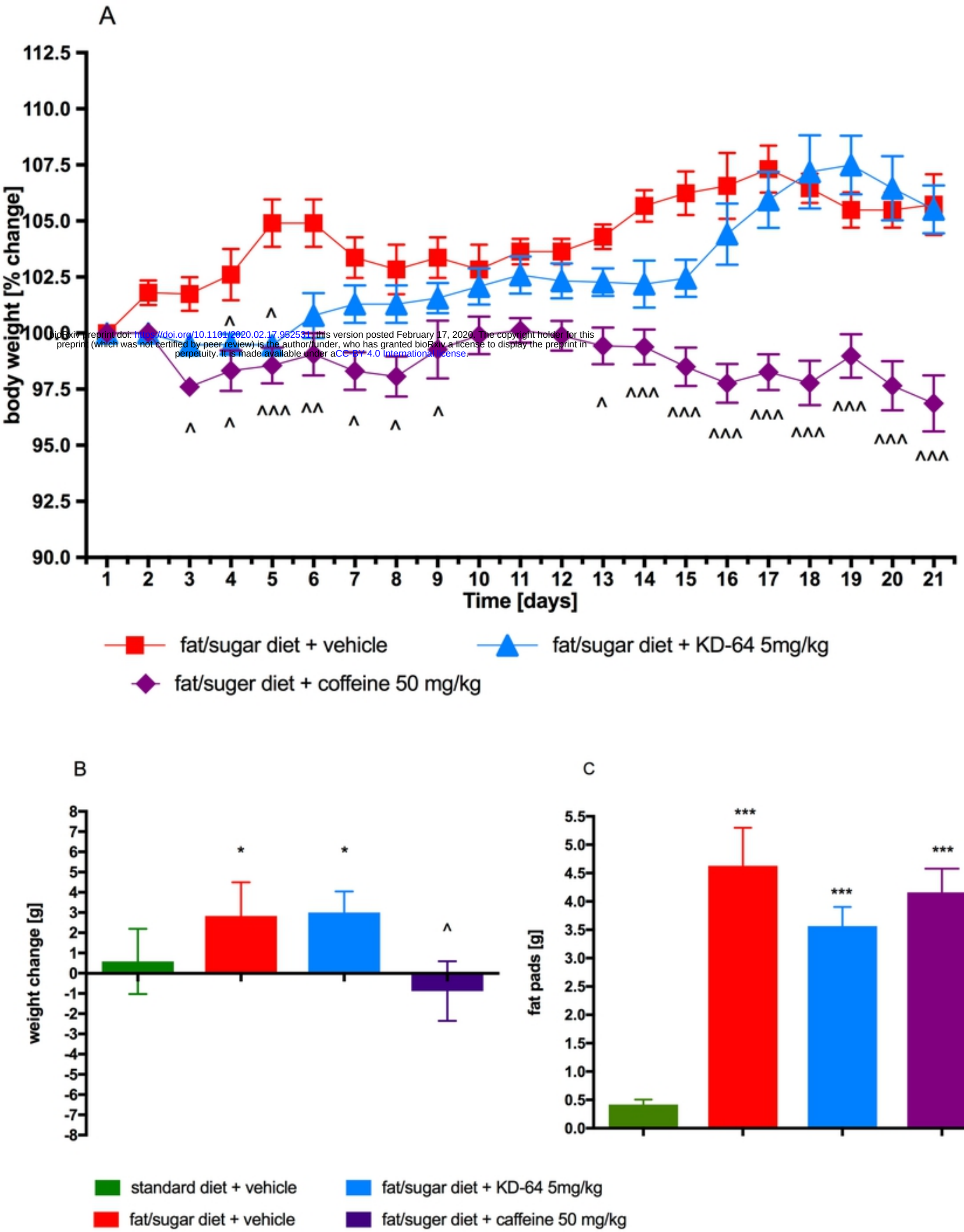
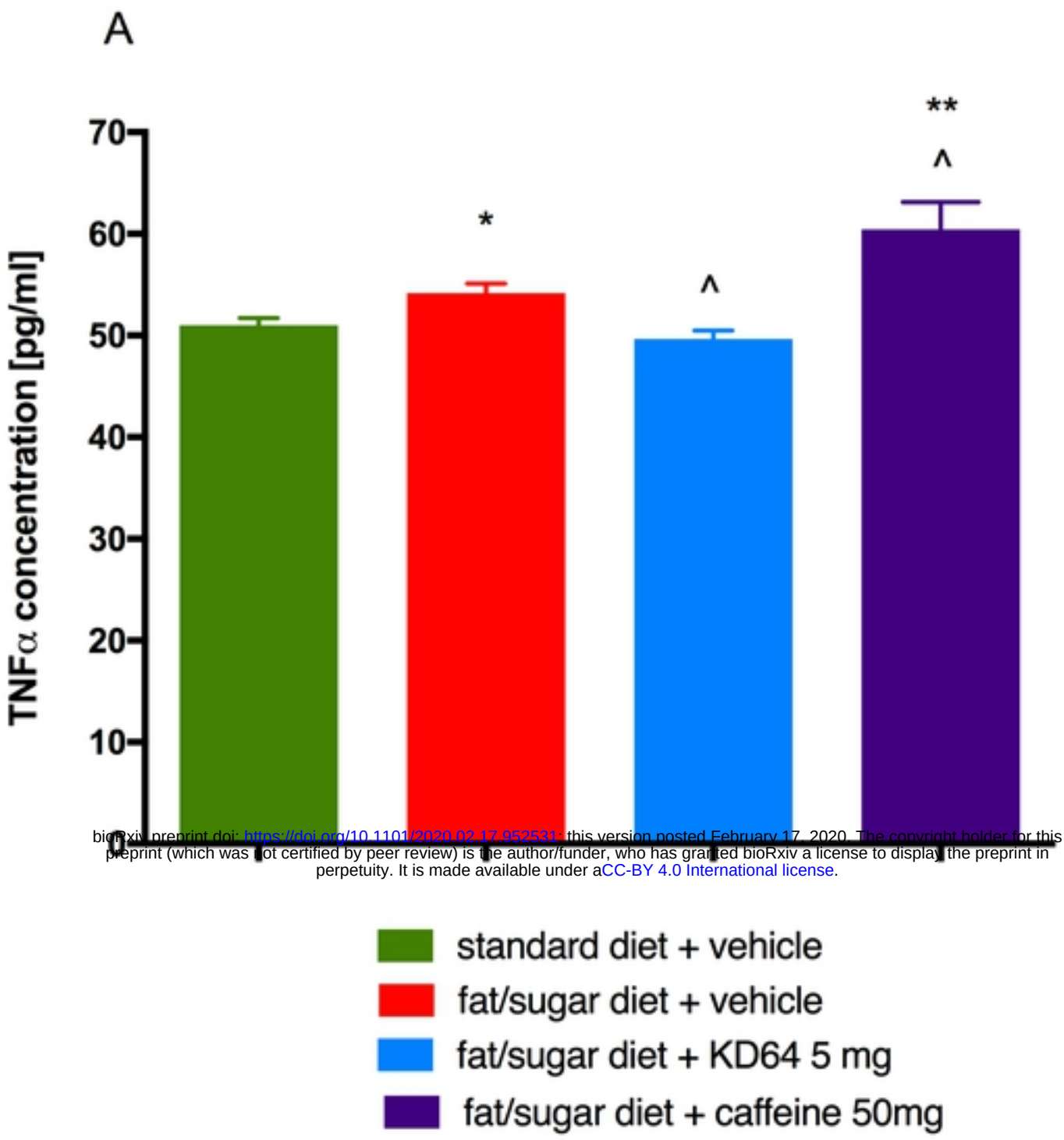


Figure 5



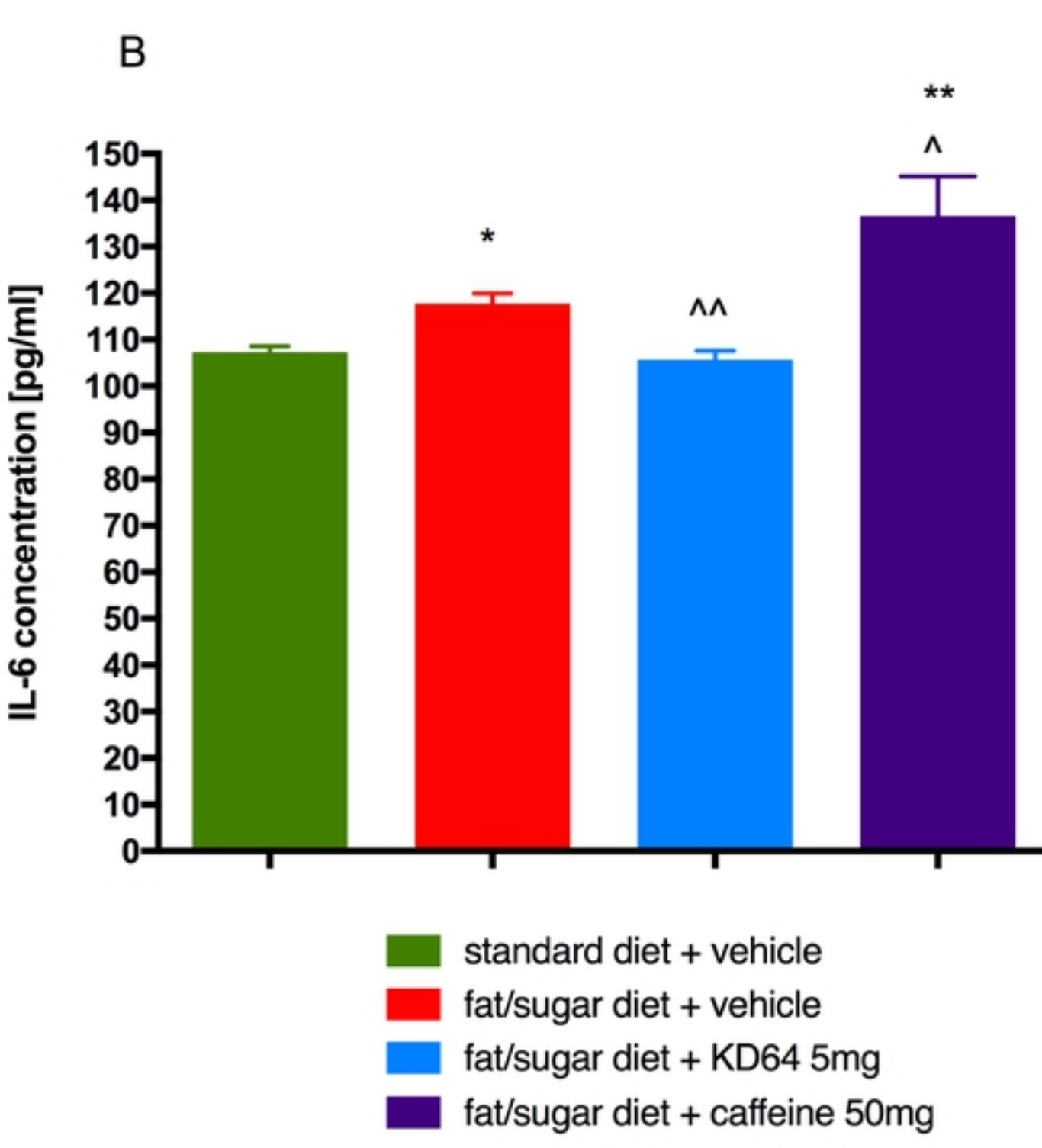


Figure 6

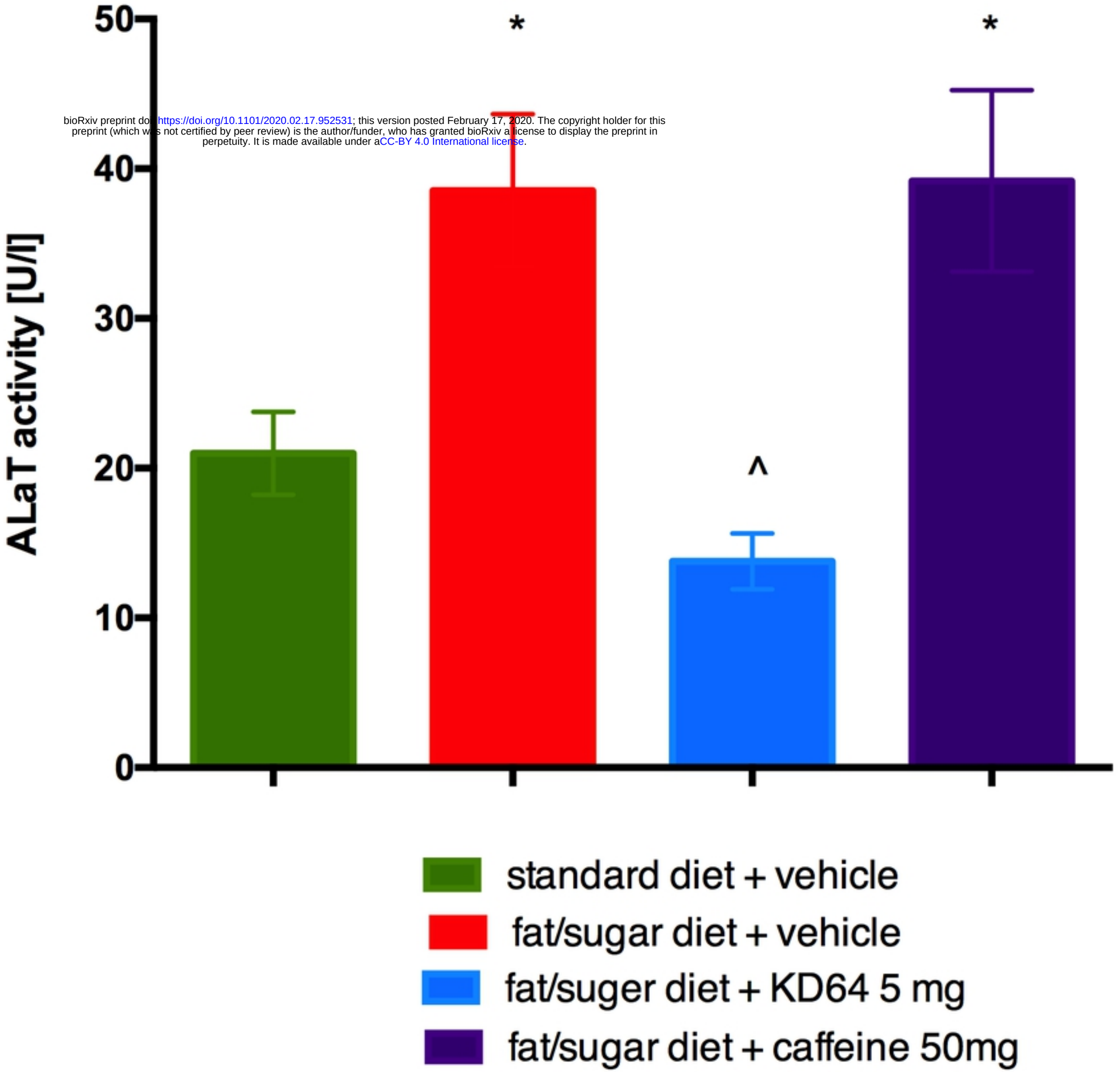


Figure 7

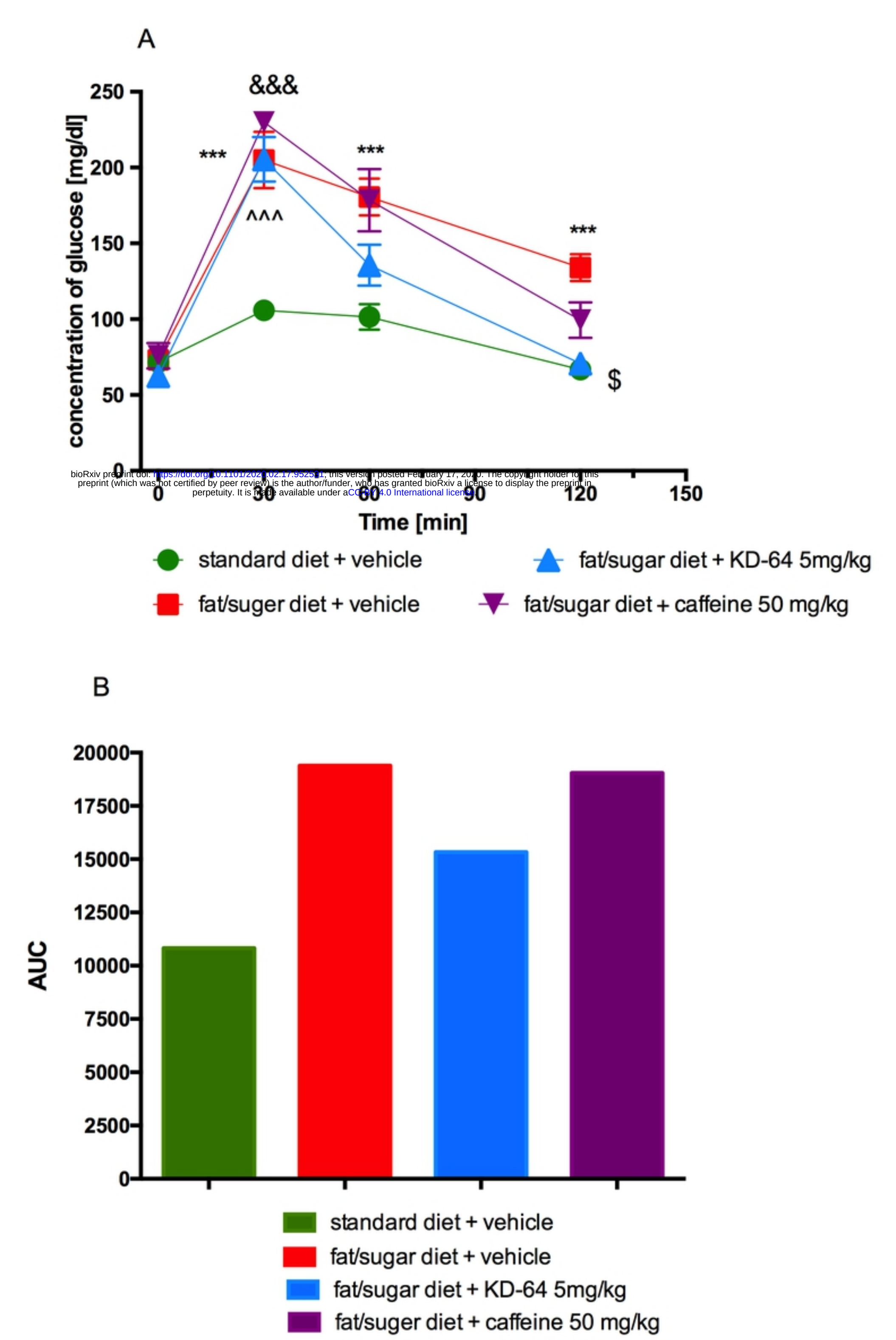
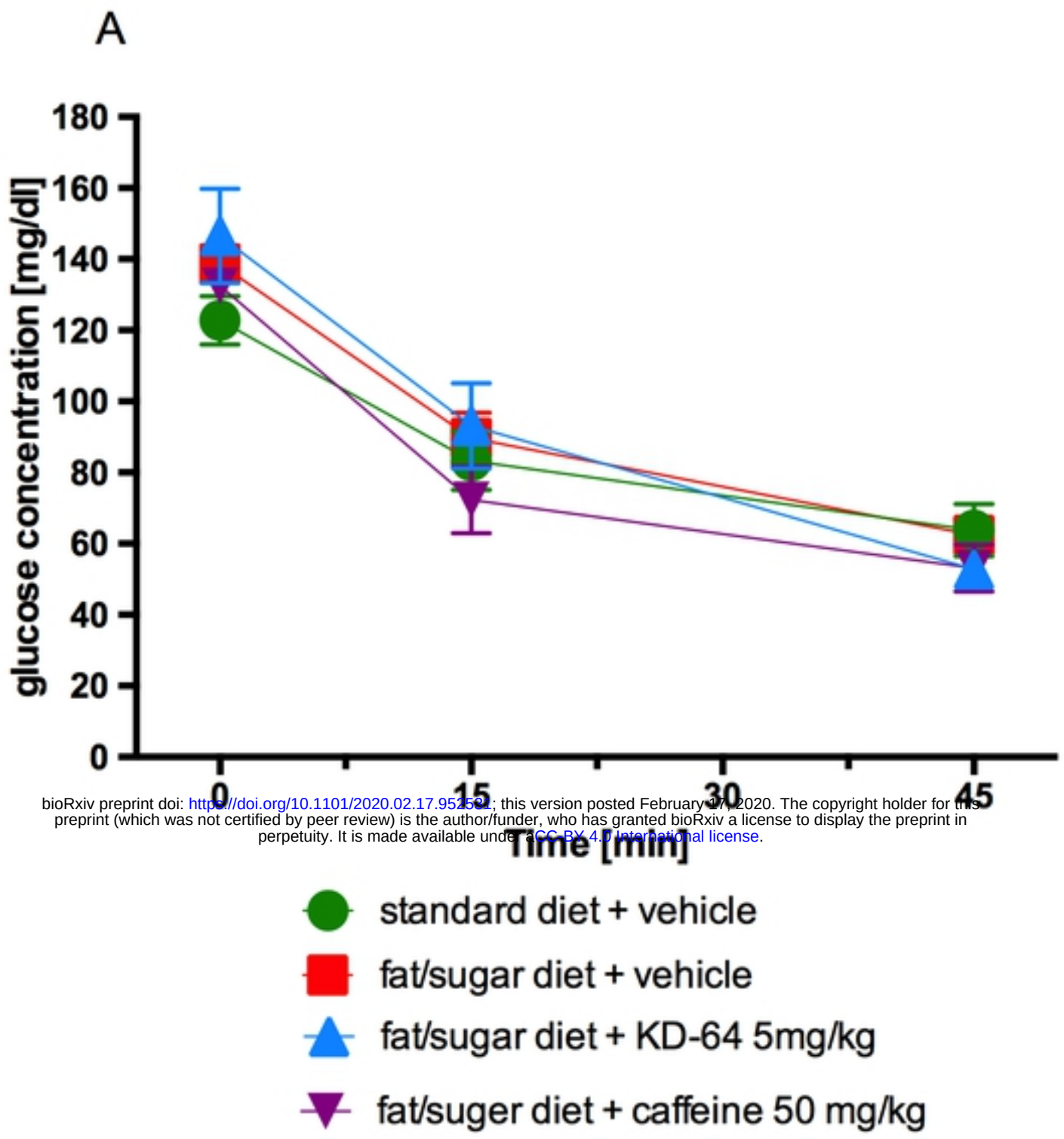


Figure 8



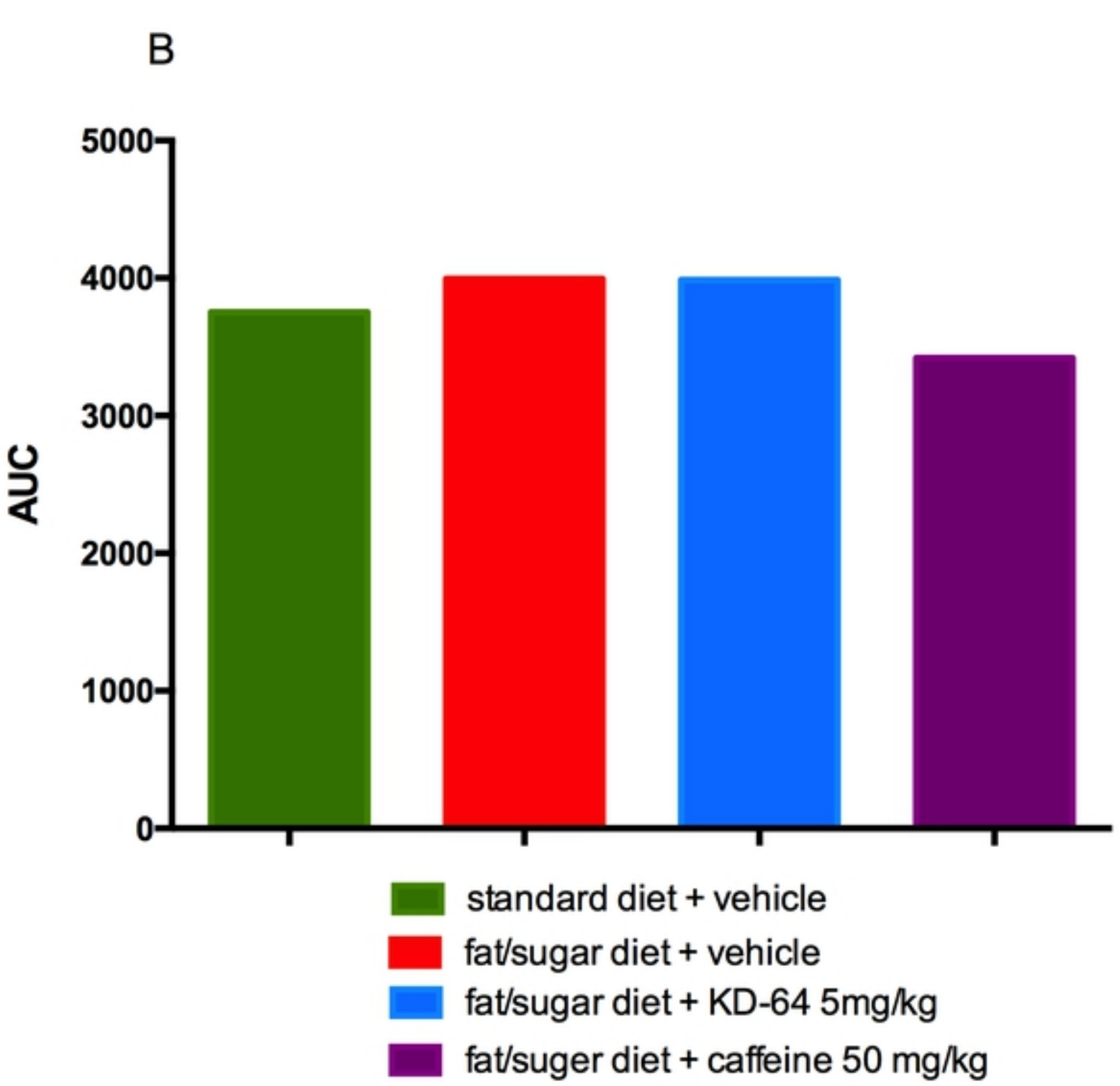
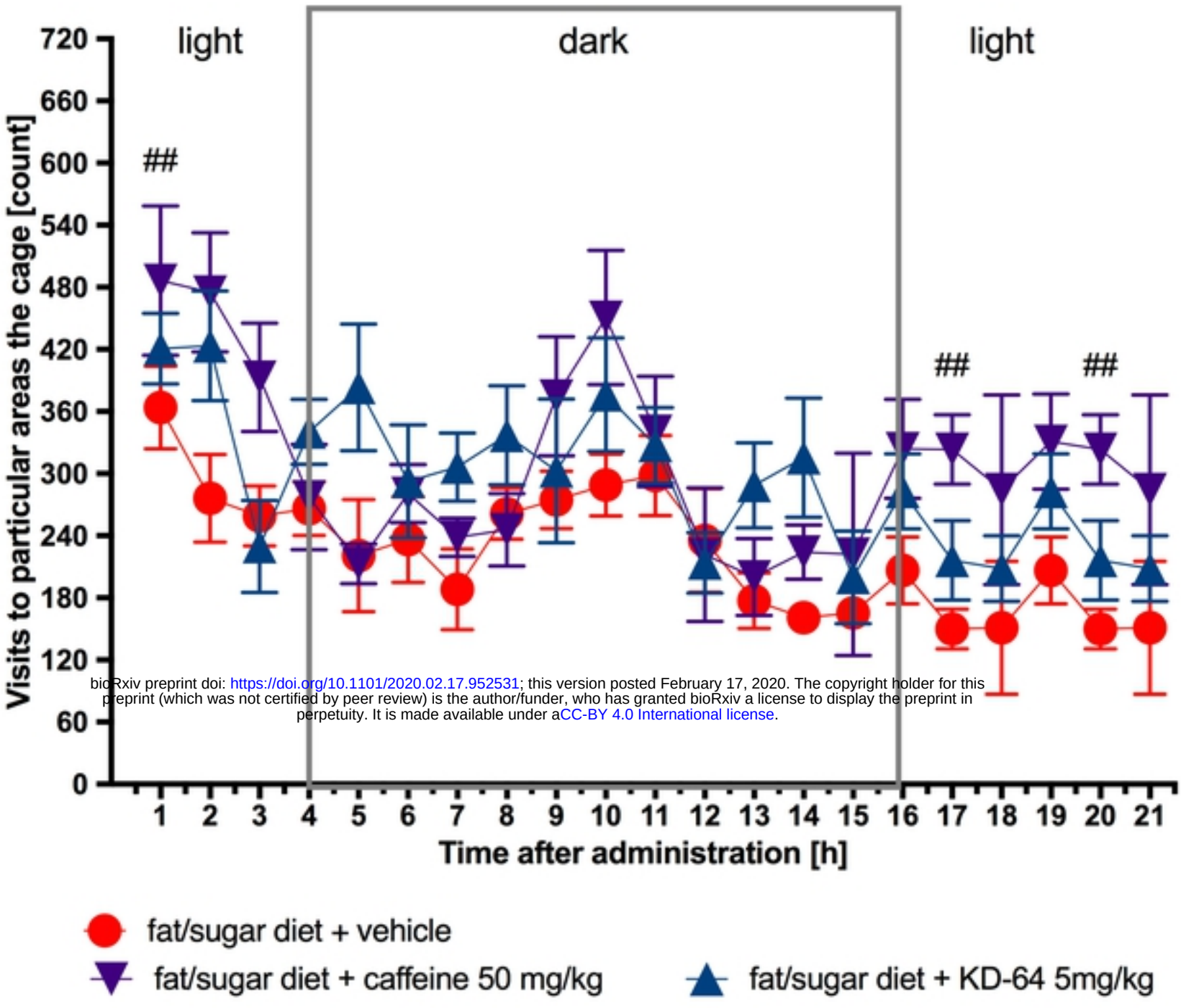


Figure 9

A



B

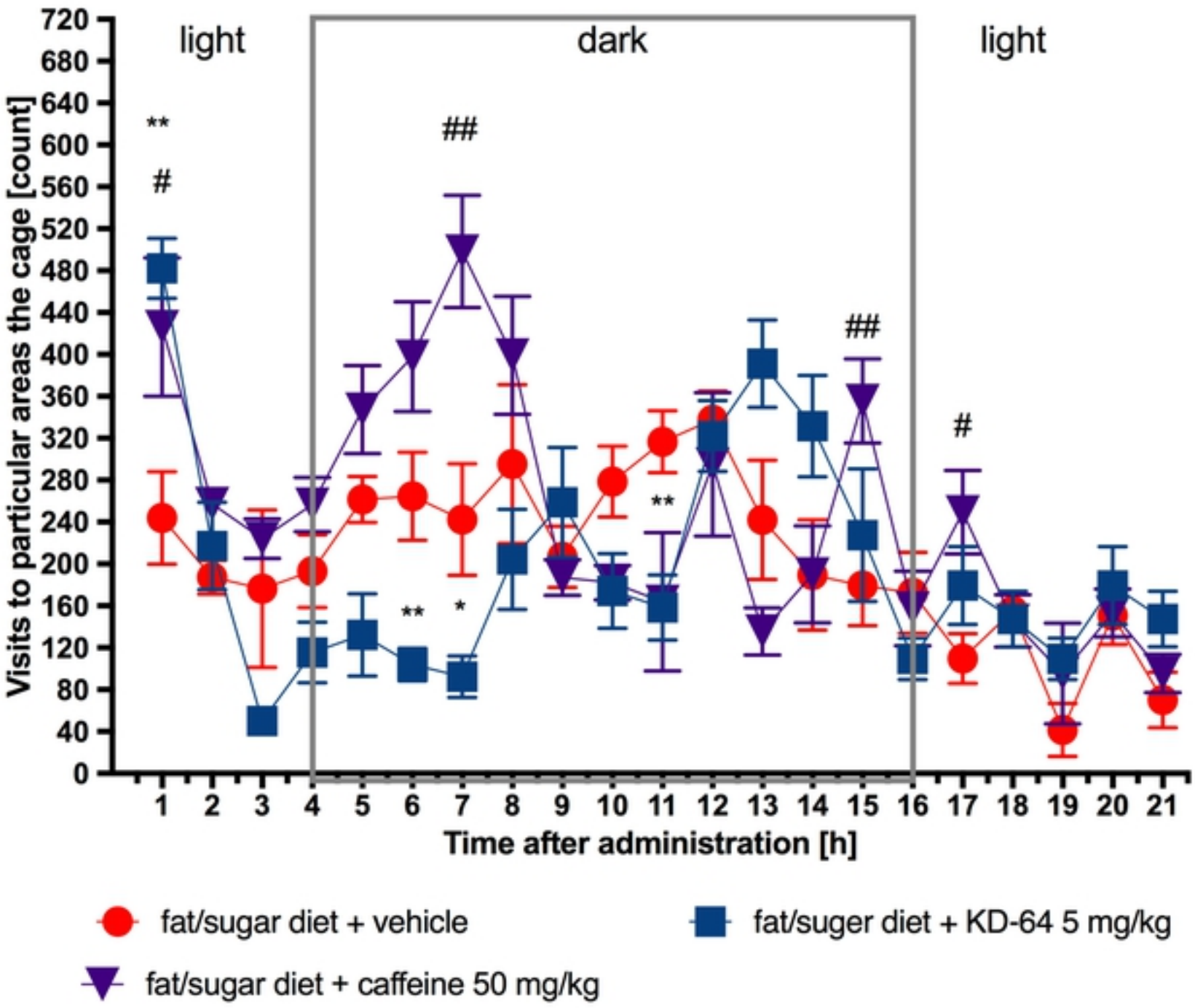


Figure 10