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Title: Metabolic and renal effects of dietary advanced glycation end products in pregnant rats – A Pilot Study

Short title: AGE-rich diet in pregnancy

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Summary

Thermally processed food contains advanced glycation end products (AGEs) including N^{ε}-(carboxymethyl)lysine (CML). Higher AGEs or circulating CML were shown to be associated with pregnancy complications such as preeclampsia and gestational diabetes. It is unclear whether this association is causal. The aim of our study was to analyze the effects of dietary CML and CML-containing thermally processed food on the metabolism in pregnant rats. Animals were fed with standard, with AGE-rich diet from gestation day 1. Third group received standard diet and gavaged CML. On gestation day 18, blood pressure was measured, urine and blood were collected and the oral glucose tolerance test was performed. Plasma AGEs were non-significantly higher in pregnant rats fed with the AGE-rich diet (p=0.09). A non-significant trend towards higher CML in plasma was found in the CML group (p=0.06). No significant differences between groups were revealed in glucose metabolism or markers of renal functions like proteinuria and creatinine clearance. In conclusion, this study does not support the hypothesis that dietary AGEs such as CML might induce harmful metabolic changes or contribute to the pathogenesis of pregnancy complications. The short duration of the rodent gestation warrants further studies analyzing long-term effects of AGEs/CML in preconception nutrition.

Key words: glycotoxins, melanoidins, Maillard reaction products, metabolic syndrome, insulin sensitivity

Introduction

Advanced glycation end products (AGEs) are formed when sugars and other aldehydes interact with proteins in the so-called Maillard reaction. AGEs are involved in several physiological processes like bone metabolism, lung homeostasis or activation of immune response (e.g. via activation of NF-κB signalling pathway) (Ott *et al.* 2014). Nevertheless, the main causes of *in vivo* AGEs formation are hyperglycemia (Goldin *et al.* 2006), oxidative stress (Tan *et al.* 2007) and aging (Brownlee 1995). Circulating AGEs levels are especially high in diabetic patients (Nowotny *et al.* 2015). Formation and recognition of AGEs by the immune system are involved in the pathogenesis of chronic complications of diabetes mellitus (Goh and Cooper 2008, Yan *et al.* 2008), chronic renal insufficiency or even neurodevelopmental disorders like those on the autism specter (Anwar *et al.* 2018).

Besides formation in vivo, AGEs are also generated during thermal processing of food, especially frying and broiling (Goldberg *et al.* 2004). The concentration of AGEs depends on the temperature, duration of thermal processing, used ingredients and type of food. Dietary AGEs are found in a wide range of processed food, but they are present also in raw foods of animal or plant origin. Thus, the amount of ingested AGEs depends on individual dietary habits, but the consumption of thermally processed foods leads to a continuous exposure to AGEs (Sebekova and Brouder Sebekova). These diet-derived AGEs have been hypothesized to induce insulin resistance and diabetes (Vlassara and Uribarri 2014), and might contribute to the development of diabetes-associated inflammation and angiopathy (Vlassara *et al.* 2002). Moreover, it has been hypothesized that AGEs-rich diet might assist in the development of the autistic traits, thus, maternal lifestyle and dietary habits might play an important role in neurodevelopment (Maher 2012, Currais *et al.* 2016). However, whether AGEs are involved in the manifestation of diabetes and other disorders or whether an impaired glucose metabolism contributes to elevated AGE concentrations can only be tested in animal experiments.

Animal experiments have shown that chronic intake of dietary AGEs induces insulin resistance (Cai *et al.* 2012), accelerates the progress of renal fibrosis (Feng *et al.* 2007), liver inflammation (Patel *et al.* 2012), might cause oxidative status impairment (Firmin *et al.* 2018) and even loss of spatial memory in a model of neurodegeneration (Lubitz *et al.* 2016). More importantly, interventional study in humans has shown that a reduction of AGEs intake improves insulin sensitivity, indicating potential preventive/therapeutic efficiency (Uribarri *et al.* 2011).

AGEs represents a heterogeneous group of compounds including, N$^{\varepsilon}$-(carboxymethylhydroxy)lysine, N$^{\varepsilon}$-(carboxyethyl)lysine and many others (Gkogkolou and Bohm 2012). N$^{\varepsilon}$-(carboxymethyl)lysine (CML) is one of the first defined as well the most prevalent AGE compound (Ahmed *et al.* 1986, Delgado-Andrade 2016). CML can be found in high amounts in various foods belonging to the so called Western style diet (Hull *et al.* 2012). Bread, and especially the bread crust, are a major source of food-derived AGEs, with CML being most abundant (Helou *et al.* 2016). Chronic exposure to CML leads to its accumulation in various organs, especially those containing long-lived proteins, such as collagen (Roncero-Ramos *et al.* 2014, Tessier *et al.* 2016a).

The most common complications of pregnancy are preeclampsia and gestational diabetes, and in both pathologies the association with AGEs has been investigated (Chekir *et al.* 2006, Harsem *et al.* 2008). Recently, plasma CML was found to be twice as high in pregnant women with gestational diabetes as in controls (Bartakova *et al.* 2016). Interestingly, complications of modeled gestational diabetes were absent in mice without the gene encoding the receptor for AGEs, suggesting that the activation by its ligands might be a part of the etiology of gestational diabetes (Ejdesjo *et al.* 2016). Similar findings have been reported in women with preeclampsia (Alexander *et al.* 2016). Plasma AGEs were found to be higher in comparison to healthy controls already in the first two trimesters and were associated with inflammatory marker (Yu *et al.* 2012). However, whether dietary AGEs are involved in the pathogenesis of common pregnancy-related complications remains unknown.

The aim of our study was to analyse the metabolic and renal effects of dietary CML administered via oral gavage and CML-containing AGE-rich food. AGE-rich food was provided in form of bread crusts in an interventional experiment of pregnant rats throughout the gestation due to its relation with pregnancy complications like gestational diabetes and preeclampsia.

Materials and methods

The experiment was performed in compliance with EU Guidelines for scientific experimentation on animals Directive 2010/63/EU, and with the approval of the Institutional ethical committee of the Institute of Molecular Biomedicine, Faculty of Medicine, Comenius University in Bratislava, Slovakia.

Design of the experiment

Twenty-eight female Wistar rats (Anlab, Prague, Czech Republic) were mated after reaching the reproductive age with their male counterparts. However, only 18 of them got and remained pregnant. The presence of sperms in vaginal smears was used to check for successful mating and considered as the first day of pregnancy. Pregnant rats were randomized into 3 groups: control group (CTRL, n = 7), advanced glycation end products group (AGEs, n = 5) and N^{ε}-(carboxymethyl)lysine group (CML, n = 6). Rats in the CTRL group had ad libitum access to standard pelleted diet (KKZ-P-M, Top Dovo, Dobrá Voda, Slovakia, Tab. 1) throughout the experiment. Animals in the AGEs and CML groups were pair-fed according to the food consumption in the CTRL group. The AGEs group received an AGE-rich diet consisting of 75% wt/wt of the control diet (Tab. 1) and 25% wt/wt bread crusts as thermally processed food as described previously (Šebeková *et al.* 2003). The energy content was 290 kcal/100 g and 390 kcal/100 g for control and AGE-rich diet, respectively (Faist *et al.* 2002, Sebekova *et al.* 2012).

Rats in the CML group received the standard diet, but were also orally gavaged with CML on a daily basis throughout the gestation. The dose of gavaged CML was calculated according to the amount of food consumed by the animals in the AGEs group (one gram of bread crusts corresponding to 0.65 mg of CML) (Faist *et al.* 2002). Rats were kept separately in plastic cages on a 12/12 hour light/dark cycle in a temperature and humidity controlled room. All groups had free access to tap water during the whole experiment.

Body weight and food consumption were monitored daily. On the gestation day 18, blood pressure was measured using the non–invasive tail cuff method (ADInstruments, Spechbach, Germany) in the morning. Thereafter, fasting blood samples (6 hour fasting) were collected from the tail vein into Microvette EDTA tubes (Sarstedt, Numbrecht, Germany). Thereafter, all rats were placed into metabolic cages for 24 hours for stool-free urine collection.

The samples were centrifuged immediately at 2000g for 10 minutes and plasma was frozen at -80 °C for further analyses. Thereafter, an oral glucose tolerance test was performed. Gestation length and the litter size were recorded at the end of the experiment. Whole experiment is described in Figure 1.

Insulin sensitivity

Insulin was measured in fasting plasma using a mammalian insulin ELISA kit (Mercodia AB, Uppsala, Sweden), according to the instructions of the manufacturer. For the oral glucose tolerance test, glucose (2 g/kg of body weight). was dissolved in tap water (500 μl) and administered orally by gastric gavage Blood glucose was measured immediately before glucose administration and again at 15, 30, 60 and 120 minutes using a standard glucometer (Accu-chek Performa, Roche, Basel, Switzerland). Glucose dynamics was evaluated using calculations of positive incremental and total area under curve (AUC). In addition, the homeostatic model assessment of insulin resistance (HOMA-IR) was calculated from fasting glucose and insulin concentrations (Matthews *et al.* 1985).

Renal function tests

The volume of urine collected in the metabolic cages was recorded. The concentration of urinary creatinine was assessed using the spectrophotometric method described by Jaffe (Jaffe 1886). The concentration of plasma creatinine was assessed using the commercially available Creatinine detection kit (Arbor Assays, Ann Arbor, Michigan, USA). Creatinine clearance was calculated. Proteinuria was analyzed using the pyrogallol red method (Watanabe *et al.* 1986), as described previously.

AGEs and CML analysis

AGE-associated fluorescence of plasma and both, control and AGEs-rich diet (10% homogenates) was determined fluorimetrically (λ_{ex} = 370 nm/λ_{em} = 440 nm), using a previously described method (Munch *et al.* 1997). For quantitative analysis, an external calibration using AGE-modified bovine serum albumin was used. The concentration of CML in plasma was analyzed using the AGE – CML ELISA kit (Microcoat, Bernried am Starnberger See, Germany), according to the instructions of the manufacturer. The concentration of total proteins was measured using the bicinchoninic acid method (Sigma-Aldrich, Munich, Germany). Bovine serum albumin was used as reference for external calibration. All measurements were performed on a Tecan Sapphire II microplate reader (Grödig, Austria). Concentration of CML in AGEs-rich diet was analyzed by reverse-phase high performance liquid chromatography, as described previously (Faist *et al.* 2002).

Statistical analysis

Power analysis was performed using G Power 3.1.9.2 (Universität Kiel, Germany). To achieve statistical power of 0.80, AGEs and CML were used as end-point variables. Preliminary data suggested expected effect size f = 0.61 and f = 0.56, respectively. The number of the animals for 3 groups was calculated to be 21 and 24, respectively (F test, ANOVA: repeated measures, between factors). However, only 18 female animals got and remain pregnant in the experiment, i.e. the final achieved power was 0.72 for AGEs and 0.64 for CML.

The data were further analyzed using GraphPad Prism 7.01 (La Jolla, California, USA). One-way ANOVA, and repeated measures ANOVA were used for the comparison of groups and for time series analysis, respectively. Bonferroni-modified t-test was used as a post-hoc test to partially prevent multiple comparison bias. Statistical significance was set to $p < 0.05$. All data are presented as mean ± standard deviation. Mischief (Made with Mischief Inc., The Foundry, London, UK) and Microsoft Office PowerPoint 2011 (Microsoft Corporation, Redmond, USA) were used for creation of artwork.

Results

Body weight of pregnant rats increased continuously during gestation (F = 40.2, $p < 0.001$). No differences were found between the groups throughout the experiment (F = 0.24, $p > 0.99$, Figure 2). Analyses of renal functions revealed differences between the groups neither for plasma creatinine (F = 0.72, p = 0.50, Figure 3a) nor for creatinine clearance (F = 1.98, p = 0.17, Fig. 3b). Similarly, no differences were observed between the groups in proteinuria (F = 0.30, p = 0.75, Figure 3c). Analysis of urine revealed high inter-individual variability in all groups. Neither AGE-rich diet nor CML administration affected? systolic blood pressure (F = 0.58, p = 0.57, Figure 4).

To assess glucose metabolism, fasting blood glucose and insulin were measured before glucose administration. Fasting glucose was 3.7 ± 0.9 mmol/l in the CTRL group. Although the average concentrations in experimental groups were slightly higher (4.3 ± 0.5 mmol/l in the AGEs group, and 4.1 ± 1.2 mmol/l in the CML group), the differences in comparison to the CTRL group were not significant (F = 0.71, p = 0.51, Figure 5a). Mean fasting insulin concentrations were 0.48 ± 0.35 μg/l in the AGEs group, 0.37 ± 0.08 μg/l in the CML group and 0.54 ± 0.38 μg/l in the CTRL group. These differences did not reach the level of statistical significance (F = 0.52, p = 0.60, Figure 5b). The calculated HOMA-IR was also similar between the groups (F = 0.53, p = 0.60, Figure 5c). These results did not statistically differ between groups probably due to already above-mentioned inter-individual variability and inability to meet the requirements for statistical power.

In the oral glucose tolerance test, the highest values of blood glucose were found 15 minutes after glucose administration and gradually declined in all three groups. The dynamics of blood glucose during the oral glucose tolerance test was significant (F = 29.4, $p < 0.001$), but no differences between groups were observed (F = 0.14, p = 0.87, Figure 6a). Total AUC (F = 0.17, p = 0.84, Figure 6b), and positive incremental AUC (F = 0.60, p = 0.56, Figure 6c) calculations confirmed the lack of significant differences between the groups in insulin sensitivity.

Measurement of AGE-associated fluorescence in the consumed food types revealed 9.6-times higher fluorescence in AGEs-rich diet (61367 arbitrary units (AU) compared to control standard diet (6421 au) ($t = 30.97$, $p < 0.001$). The average AGE-associated fluorescence of plasma samples was 21% higher in the AGEs group, and 30% higher in the CML group in comparison to control animals. The differences were not significant ($F = 2.80$, $p = 0.09$, Figure 7a). Assessment of plasma CML concentrations showed a trend towards higher values in the AGE (by 28%) and CML (by 57%) groups compared to the CTRL group ($p = 0.06$). The difference between the groups was found to be marginally non-significant ($F = 2.93$, $p = 0.08$, Figure 7b).

The observed gestational length and the litter size at delivery were similar between the groups ($F = 0.75$, $p = 0.49$ and $F = 1.23$, $p = 0.31$, respectively; Figure 8,). The number of delivered pups was 10 ± 2.7 for the CTRL group, 11 ± 1.8 for the AGEs group and 9 ± 2.4 for the CML group.

Discussion

AGEs can be found in high amounts in numerous commonly consumed foods (Goldberg *et al.* 2004). Dietary AGEs seem to worsen or even induce complications of several chronic diseases (Luevano-Contreras and Chapman-Novakofski 2010). As AGEs are linked to gestational diabetes (Pertynska-Marczewska *et al.* 2009) and preeclampsia (Coffeng *et al.* 2011, Chen *et al.* 2016), it is reasonable to assume that dietary AGEs might also contribute to the etiopathogenesis of these most common pregnancy-related complications. In our controlled feeding experiment, high AGEs/CML intake throughout gestation did not affect metabolic outcome measures in pregnant rats. Neither renal functions nor insulin sensitivity were affected. Weight gain during gestation, and litter size were also not different between control and experimental groups.

The lack of significant differences in plasma AGEs and CML does not affect the relevance of the other results since the foods differed significantly in their AGEs content. The daily dose of consumed AGEs and gavaged CML was similar or even higher in comparison with other animal studies (Alamir *et al.* 2013, Roncero-Ramos *et al.* 2013, Poulsen *et al.* 2016) and much higher than the amount of AGEs in commonly consumed meals used in human studies (Birlouez-Aragon *et al.* 2010). Inconsistent outcomes of AGE feeding studies can be explained by varying digestive and microbial degradation of dietary AGEs in the gut, their poor absorption, and renal excretion of the absorbed AGEs (Forster *et al.* 2005). In other studies, the excreted CML was found in the urine and faeces (Roncero-Ramos *et al.* 2013). The concentration of excreted CML in stool correlated with the level of ingested CML. Absorbed CML was found in the heart and tail tendon. Unfortunately, such analyses have not been performed in our study. However, similarly to our study, protein bound CML as well as free CML in plasma was not elevated in the groups with AGEs-rich diet when compared to low AGEs diet (Alamir *et al.* 2013, Roncero-Ramos *et al.* 2013).

While some studies clearly show negative health effects even after a bolus administration (Koschinsky *et al.* 1997), other human studies have found no effects of 6 weeks-long high intake of AGEs (Semba *et al.* 2014). Practical guidelines to reduce the intake of AGEs are already published (Uribarri *et al.* 2010). However, a systematic review reported that there is insufficient evidence for recommending an AGE-restricted diet (Kellow and Savige 2013). In the recent study, even an increase of insulin sensitivity has been observed in mice fed a CML-containing Western diet for 8 weeks (Ward *et al.* 2016). Clearly, more research is needed in this area.

A major limitation of our experiment is the low number of animals in the individual groups. Next, the duration of rodent pregnancy is much shorter than in humans. Also, rats used in this study were mating shortly after reaching the adulthood, thus, they were quite young to manifest profound age-related AGE-modification of proteins. Considering the duration of pregnancy, pregnant women would be exposed to the AGEs-rich diet much longer n human interventional study in comparison with rodents. However, it would be difficult to perform a similar clinical study. In general, human diet is more complex, thus, it would not be possible to ensure analogous diet necessary for this type of study for such a long time period, especially during pregnancy. On the other hand, observational studies comparing women consuming high- vs. low-AGEs diets would be biased by additional nutritional, metabolic, and other differences between the groups.

Nevertheless, studies regarding diet performed on rodents have been already published (Ruhlen *et al.* 2011, Zhang *et al.* 2013). Our results are in line with a recently published study in non-pregnant rats, showing no metabolic consequences of short-term dietary AGE intake (Poulsen *et al.* 2016). The lack of significant differences in plasma AGEs or CML between the groups in our study could be explained by their low and fast absorption from food (~10%), excretion by kidneys (~30%) (Gugliucci and Bendayan 1996, Koschinsky *et al.*

1997), their storage in the tissues (Tessier *et al.* 2016b) or by the low number of animals and high variability. Also, it is likely that AGEs accumulated in the tissues rather than in plasma. Lastly, we measured total and not protein-bounded CML as recommended by others (Roncero-Ramos *et al.* 2013). In pregnant women, even a single oral glucose load has been shown to increase plasma CML (Williams *et al.* 2012). Whereas we did not observe differences in fasting glucose or glucose dynamics during the oral glucose tolerance test, variability of blood glucose was not assessed during the whole gestation period. However, experimental studies performed on rats showed that the concentration of glucose, glycated hemoglobin and fructosamin decrease by the end of the gestation (Preston *et al.* 1992).

This might introduce a bias, contributing to the lack of significant differences in plasma AGEs/CML despite clearly higher intake of AGEs/CML. Nevertheless, these technical limitations do not affect the major negative findings of our experiment. Especially, while the daily dose of CML in our experiment was similar to that administered in other animal experiments (Li *et al.* 2015), and substantially higher than dietary AGEs-load in clinical studies focusing on the effects of AGE-rich diets (Poulsen *et al.* 2014). Our former studies showed that prenatal oral challenge with dietary AGEs exert postnatal effects in offspring, but these studies focused on the offspring rather than the pregnant mothers (Gurecka *et al.* 2015, Csongova *et al.* 2018).

There is another study confirming the harmful effect of dietary AGEs performed by Cai et al. on mice (Cai *et al.* 2012). They showed that consumption of AGEs in form of diet enriched with AGEs precursor methylglyoxal throughout the life had negative consequences on the following generations proven by the lower insulin sensitivity, higher leptin level, lower adiponectin and higher plasma/white fat CML and methylglyoxal concentrations. Interestingly, they observed no effect of the AGEs-rich diet on the body weight gain.

Moreover, higher body weight was observed in the mice fed with standard diet without AGEs.

Results observed by Cai et al. point to the consequences of epigenetics and overall eating habits on the offspring. On the other hand, AGEs-rich diet used in this study was not prepared naturally by exposure to high-temperature but with adding of a synthetically prepared methylglyoxal. The effect of ingested reactive AGE-precursor might differ from that of dietary (naturally formed) AGEs.

According to our knowledge, this is the first experiment studying the metabolic and renal effects of dietary AGEs in pregnant animals. Despite negative findings and several limitations, our results are of importance, since this experiment is the first to test whether high intake of AGEs/CML induces features of gestational diabetes or preeclampsia. One of the potential explanation of the lack of observed metabolic or renal effects might be that the animals were on a standard diet, while high-fat diet has been shown to increase CML absorption (Davis *et al.* 2015). Based on previously published research, we hypothesized that AGEs might induce embryonal toxicity (Hao *et al.* 2008). In addition, one other study showed that preexisting maternal diabetes leads to accumulation of AGEs, including CML already in the blastocyst (Haucke *et al.* 2014). However, as neither the weight gain during pregnancy, nor the litter size were affected by dietary AGEs/CML, the results presented here do not support our hypothesis. On the other hand, if the treatment with dietary AGEs would have started earlier, i.e. before conception, our results might have been different.

In conclusion, our experiment does not support the hypothesis that high dietary intake of AGEs/CML, if limited to the period of pregnancy, induces or participates on the pathogenesis of pregnancy-related complications namely high blood pressure, proteinuria or gestational diabetes generally known as preeclampsia. More likely, the observed higher AGEs/CML in pathological pregnancies might rather be a consequence than a cause of the disease pathomechanisms (Bartakova *et al.* 2016). Taking into account the short duration of our experiment and the healthy status of the pregnant rats, our results do not exclude the possibility that high dietary AGEs/CML intake might induce negative effects in human pregnancies or in pregnant animals with already established insulin resistance, diabetes or kidney diseases. Further studies should focus on the effects of long-term intake of dietary AGEs/CML, especially during the preconception period.

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Conflict of interest

There is no conflict of interest.

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Tables

Table 1: Composition of the control feeding diet

Ingredients	**Proportion**
Wheat	50%
Meat and bone meal	11%
Oats	8%
Barley	8%
Alfalfa	6%
Extruded soybeans	6%
Wheat bran	4%
Wheat sprouts	4%
Mineral additives	1.4%
Sunflower oil	1%
Vitamin premix	0.6%

Figures

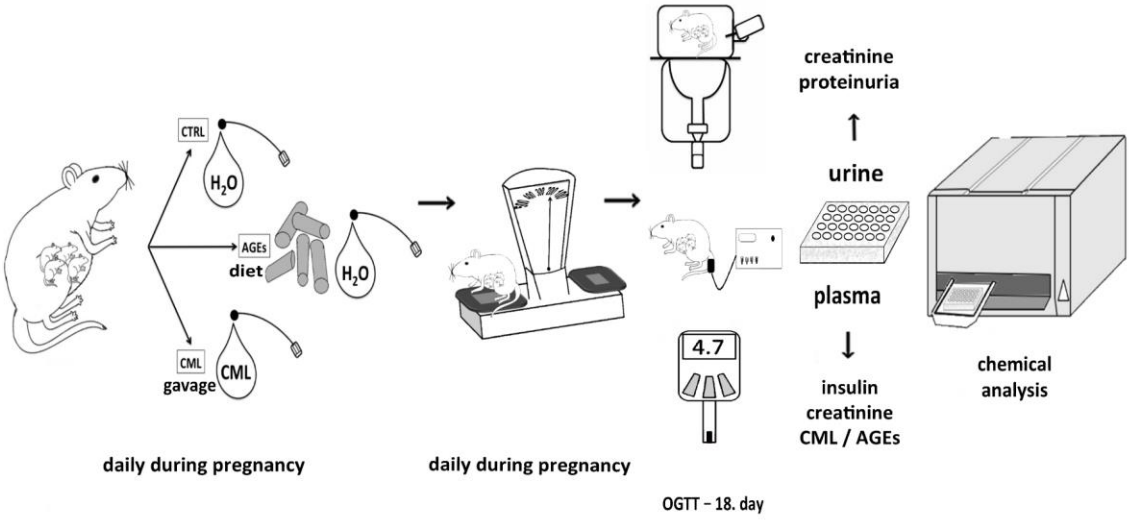

Figure 1. Design of the experiment.

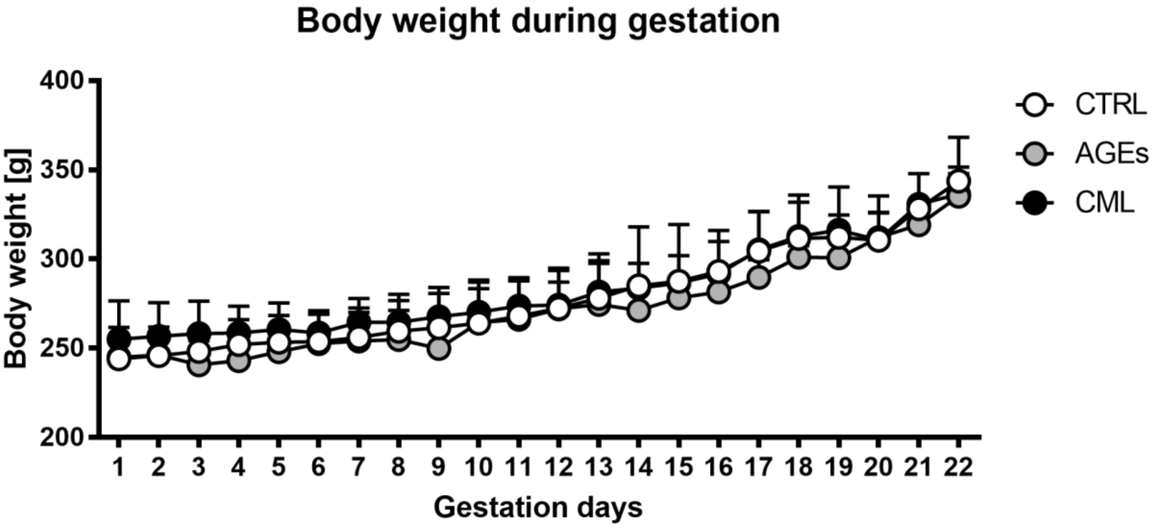

Figure 2. Body weight dynamics. The weight of the pregnant rats was monitored daily throughout the whole gestation. Data are presented as the mean + SD. (we visually changed the Y axis on the graph according to A reviewer´s suggestion)

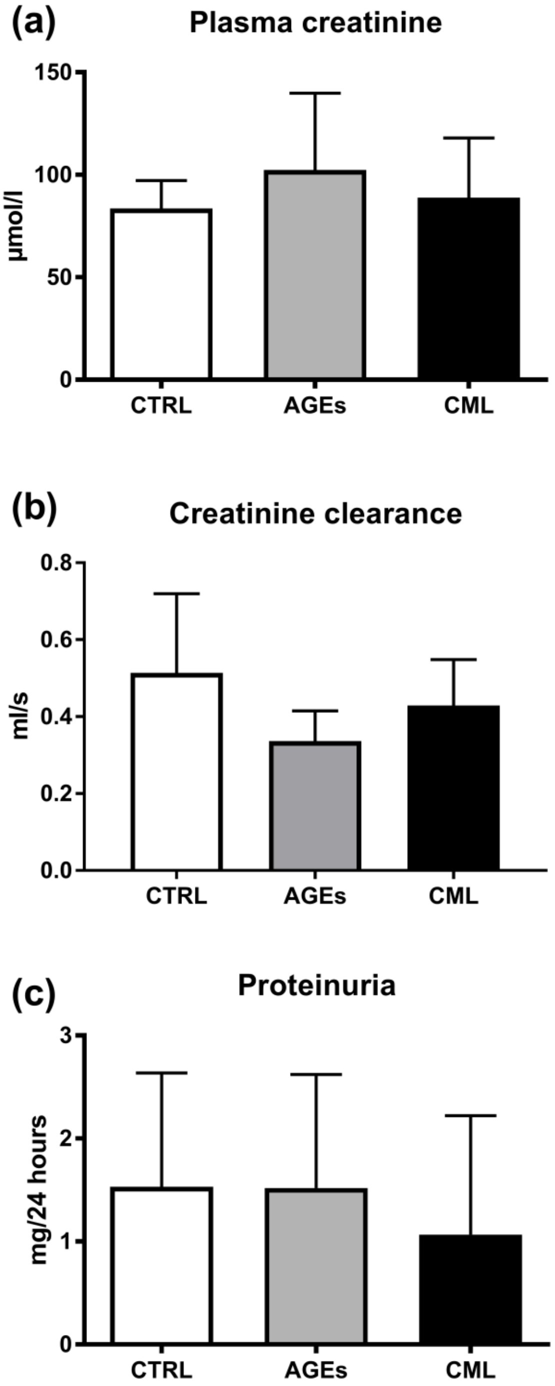

Figure 3. Renal functions. Plasma creatinine (*a*), creatinine clearance (*b*) and proteinuria (*c*) were measured at the end gestation and did not differ between the groups. Data are presented as the mean + SD. (we changed the units of the graphs (*a*) and (*b*) according to B reviewer´s comment and suggestion)

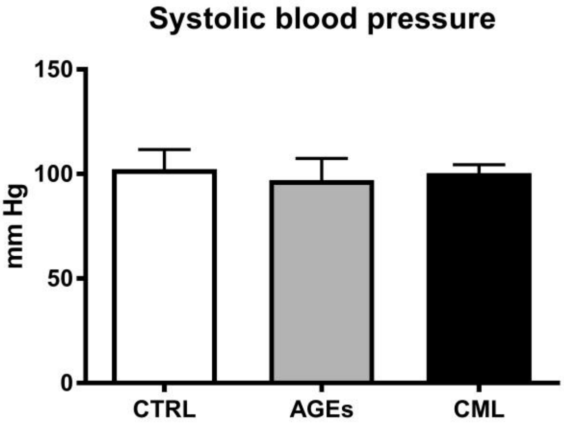

Figure 4. Systolic blood pressure. Non-invasive tail-cuff method was used for the single blood pressure measurement assessed on the 18th day of the gestation. No differences between the groups were found. Data are presented as the mean + SD.

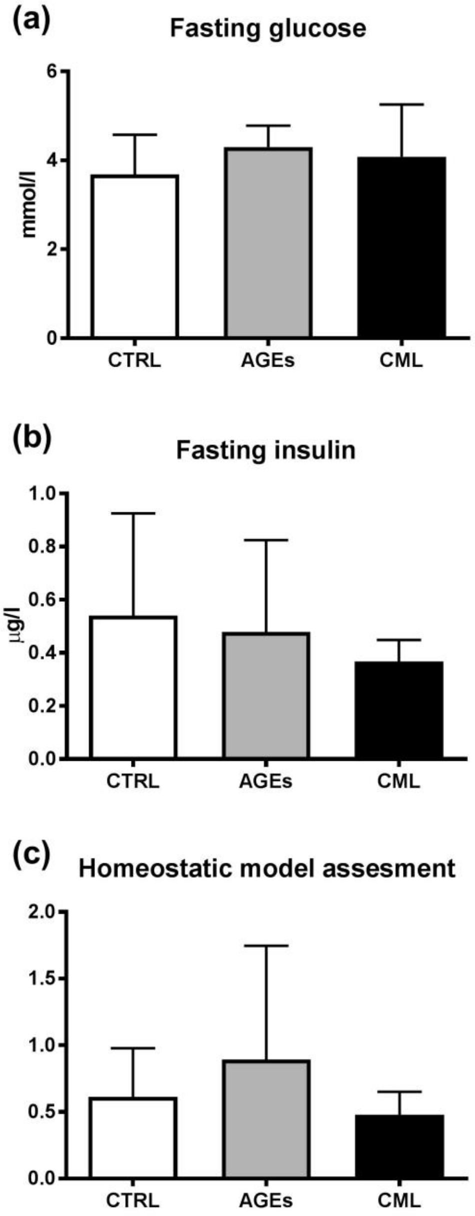

Figure 5. Insulin sensitivity. In fasting blood samples the concentrations of glucose were assessed (*a*). Fasting insulin was measured using ELISA in plasma samples (*b*). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated (*c*). No differences in insulin sensitivity were found between the groups. Data are presented as the mean + SD.

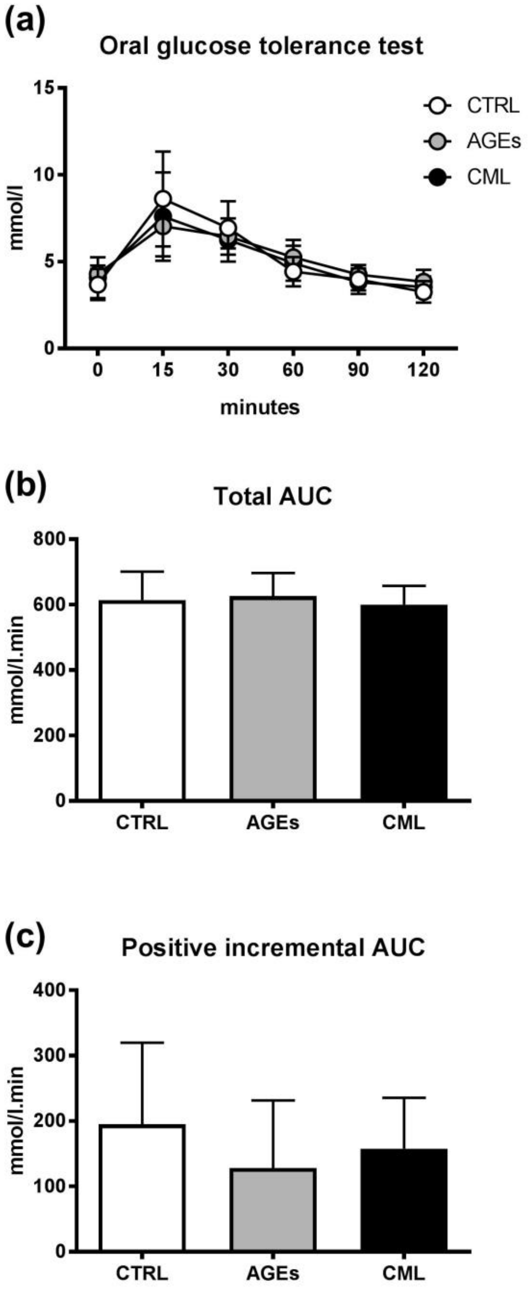

Figure 6. Oral glucose tolerance test. The dynamics of blood glucose concentrations was observed before and after glucose administration via oral gavage (*a*). Based on the glucose dynamics total (*b*) and positive incremental (*c*) areas under curve were calculated. Data are presented as the mean + SD.

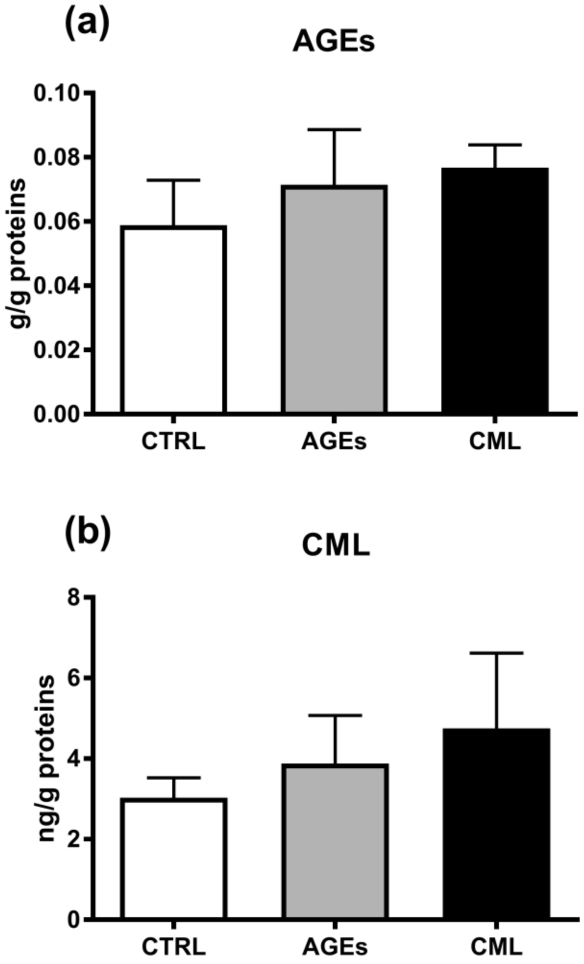

Figure 7. In collected plasma samples advanced glycation end products (AGEs) were assessed using AGE-specific fluorescence (*a*). ELISA kit was used for the measurement of N^{ε}-(carboxymethyl)lysine (CML, *b*). The concentrations were normalized to total plasma proteins. No significant differences between groups were found. Data are presented as the mean + SD.

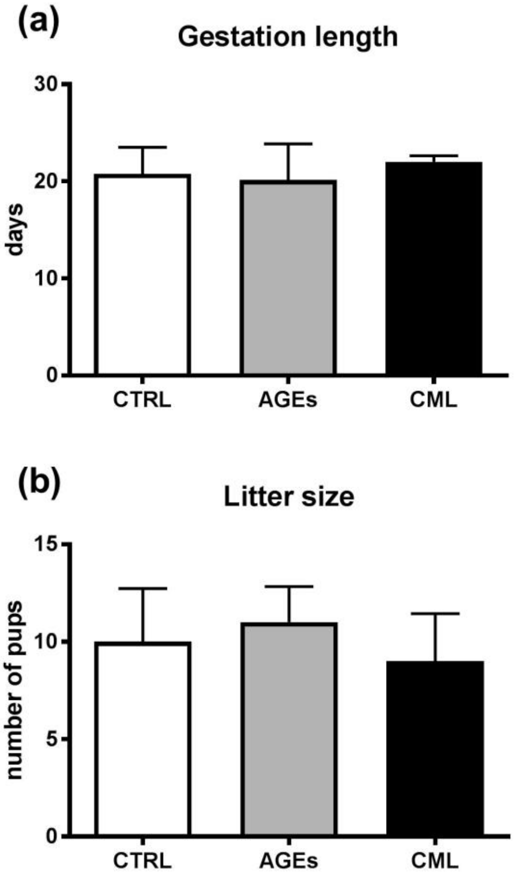

Figure 8. Gestation length and litter size. After sample collection, rats were allowed to deliver. The day of delivery and, thus, the gestation length was monitored (*a*). In addition, the number of pups was recorded for every rat as litter size (*b*). No differences between the groups were found. Data are presented as the mean + SD