Pepsin egg white hydrolysate ameliorates metabolic syndrome in high-fat/high-dextrose fed rats.

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Abstract

The aim of this study was to examine the effect of a pepsin egg white hydrolysate (EWH) on metabolic complications using a high-fat/high-dextrose diet-induced Metabolic Syndrome (MetS) experimental model. Male Wistar rats where divided in 4 groups which received: standard diet and water (C), standard diet and a solution with 1g/kg/day of EWH (CH), high-fat/high-dextrose diet and water (MS), and high-fat/high-dextrose diet and a solution with 1g/kg/day of EWH (MSH). EWH consumption normalized body weight gain, the abdominal obesity and the peripheral neuropathy developed in MetS animals, reduced adipose tissue and liver weight, as well as plasma glucose. Oxidative stress and inflammation biomarkers were normalized in MSH animals. In conclusion oral administration of EWH could be used as a functional food ingredient to improve some complications associated to MetS induced by unhealthy diets.

Keywords

Egg white; hydrolysate; bioactive peptides; metabolic syndrome; diet induced obesity; rat model.

1. Introduction

Metabolic syndrome (MetS) is a complex disorder which refers to the clustering of central obesity, insulin resistance, impaired glucose tolerance, hypertension and dyslipidemia.[1,2] This pathology increases the risk to develop diabetes, cardiovascular diseases, non-alcoholic fatty liver disease and microvascular complications including peripheral painful neuropathy and/or autonomic neuropathy.[3] The prevalence of MetS is increasing fast, especially in developing areas undergoing rapid socio-environmental changes.[4] One of the major causes of obesity is a diet rich in both, sugar and saturated fat.[4,5] This diet, known as "Western diet", leads to disturbances in carbohydrate and lipid metabolism that promotes metabolic complications.[6]

The current treatment used in MetS complications are lifestyle change interventions, pharmacotherapy and, in some cases, surgery, being a dietary intervention probably the safest and most cost-effective option. Along this line, various studies have emphasized the possibility of using food-derived compounds as natural ingredients to control metabolic complications related to MetS.[7,8] Bioactive peptides are released during food processing or after digestion of food proteins from different sources (milk, egg, rice, fish…) and they can exert different biological activities. Some of them may help metabolic syndrome conditions.[9] In this context, egg derived peptides have demonstrated angiotensin converting enzyme (ACE) inhibitory activity,[10,11] antioxidant activity,[12,13] antihypertensive effects after short[14] and long term administration,[15] and beneficial properties on the lipid profile of spontaneously hypertensive rats (SHR).[16] Moreover, our research group has obtained an hydrolysate

from egg white which simultaneously possess antioxidant, hypocholesterolemic and DPP-IV inhibitory activities, both *in vitro* and *in vivo* in Zucker fatty rats.[17,18]

Currently, high-fat/high-carbohydrate diet-induced MetS is one of the most relevant animal models to mimic the diet responsible for human MetS as a basis to investigate its potential interventions.[19,20] High-fat/high-carbohydrate diets induced in rats most of the symptoms of MetS such as hypertension, dyslipidemia, impaired glucose tolerance, excess fat deposition, increased proinflammatory markers and oxidative stress and also peripheral polyneuropathy.[1,21]

The aim of this study was to examine the effect of a pepsin egg white hydrolysate (EWH), previously characterized in our research group,[17] on metabolic complications related to MetS developed in high-fat/high-dextrose diet-induced MetS rats.

2. Material and methods

2.1. Preparation of egg white hydrolysate

The EWH as carried out according to the method of Garcés-Rimón et al.[17] Briefly, pasteurized egg white was hydrolysed with food grade pepsin from pork stomach (E.C. 3.4.23.1. BC PEPSIN 1:3000 Biocatalysts, United Kingdom). The egg white was acidified with concentrated food grade HCl 37% (Panreac Quimica S.L.U., Spain) to pH 2. The samples were incubated at 37 °C under constant stirring in a thermostatic water bath for 8 hours. Inactivation of pepsin was achieved by increasing the pH to 7.0 with food grade NaOH 10M (Panreac Quimica S.L.U.). The hydrolysate was centrifuged for 15 min at 4500 g, and the supernatant was stored at -20 °C until analysis.

86 *2.2. General protocol in animals*

87 The experiments were designed to minimize the number of animals used and
88 performed in accordance with the European and Spanish legislation on care and use of
89 experimental animals (210/63/UE; Real Decreto 53/2013), and were approved by the
90 Ethics Committee at University Rey Juan Carlos (URJC).

91 Thirty-four 8-week old Wistar male rats weighting 280-310 g purchased from
92 Harlan Laboratories (Harlan Ibérica Barcelona, Spain) were used in this study. During
93 the experimental period the animals were maintained in a temperature-controlled
94 room (23 °C), 12 h light/dark cycles and *ad libitum* access to water and feed.

95 The rats were randomly divided into 4 groups which were fed, for 20 weeks,
96 with standard chow diet (A04, SAFE, France) and tap water (C, n=7), standard chow
97 diet and an EWH solution 1g/kg/day (CH, n=7), high-fat diet (Purified Diet 235 HF,
98 SAFE, France) with a 25% dextrose solution (MS, n=10) and high-fat diet with a 25%
99 dextrose and an EWH solution 1g/kg/day (MSH, n=10). The EWH was provided from
100 the week 10th until the week 20th of the study. The daily doses of 1 mg/kg were
101 selected according to the results obtained after *in vitro* studies[17] and from previous *in*
102 *vivo* studies using EWH in SHR.[14-16]

103 During the experimental period, the body weight of the animals was recorded
104 weekly up to the 20th week of the study. Drinking fluids and food intake were
105 estimated weekly from the different groups. The occurrence of neuropathic sign
106 (tactile allodynia) was assessed once every 6 weeks using the Von Frey hair test.

107 At the end of the study, and after 16 hours of fasting, the abdominal
108 circumference and body length (nose-to-anus length) were determined in all studied
109 animals.
110 The rats were anaesthetized with an intraperitoneal injection of ketamin (87
111 mg/kg) and xilacin (13 mg/kg) and sacrificed by decapitation. Blood was collected into
112 tubes containing lithium heparin as anticoagulant. These samples were centrifuged at
113 500G for 20 minutes at 4 °C to obtain plasma which was divided into aliquots and kept
114 frozen at -80 °C until analysis. Epididymal adipose tissue, liver and tibia were
115 immediately excised. Adipose tissue and liver were weighed and tibia length was
116 registered.
117
118 *2.3. Diabetic neuropathy evaluation. Von Frey test.*
119 The development of peripheral neuropathy was evaluated with the Von Frey
120 hair test. In this test, a significant decrease in Von Frey hairs withdrawal threshold
121 evoked by tactile-mechanical stimuli is suggestive of mechanical allodynia (increased
122 sensitivity to non-noxious stimuli).
123 Mechanical sensitivity was assessed at week 0, 6, 12 and 18. Rats were placed
124 individually on an elevated iron mesh in a clear plastic cage and were allowed to adapt
125 to the testing environment for at least 10 min. Habituation to this environment was
126 also performed on the day before assessment. Calibrated Von Frey hairs ranging from
127 4 to 60 g (4, 8, 10, 15, 26 and 60 g) were applied to the plantar aspect of each hind
128 paw, from below the mesh floor. This protocol was repeated five times with 3 s
129 intervals. Withdrawal responses to the stimulus were recorded. A positive result was

considered when at least three of five responses were obtained with each filament, and this value was considered as the tactile threshold. When less than three positive responses were detected with any of the hair trials, the process was repeated with the next higher force hair.

2.4. Plasma leptin and adiponectin

Plasma leptin and adiponectin concentrations were determined using rat ELISA kits (Cusabio, BioNova científica S.L., Spain) according to the manufacturer instructions. Results were expressed as ng leptin/mL plasma and as µg adiponectin/mL plasma.

2.5. Oxidative stress biomarkers

Plasma antioxidant capacity: Antioxidant activity was determined by the oxygen radical absorbance capacity (ORAC) assay previously reported by Manso et al.[16] ORAC values were quantified by a fluorimeter (FLUOstar Optima, BMG Labtech GmbH, Germany) with wavelength excitation at 485 nm and wavelength emission measured at 520 nm. Results were expressed as µmol of trolox (Sigma, USA) equivalent/µL of plasma.

Plasma malondialdhehyde: Levels of plasma malondialdehyde (MDA) were measured by the thiobarbituric acid assay at 535 nm, using a microplate reader (Infinite M200, Tecan, Switzerland) as previously described Manso et al.[16] Results were expressed as nmol MDA/mL plasma.

Liver glutathione determination: Reduced glutathione (GSH) levels were determined by the monochlorobimane fluorimetric method previously described by Kamencic et al.[22] using a microplate reader (Infinite M200, Tecan) with wavelength excitation at 390nm and wavelength emission measured at 510 nm. Results were expressed as µmol GSH/g protein.

2.6. Glucose metabolism determinations

Plasma glucose levels were analyzed using a glucose-oxidase enzymatic commercial kit (Spinreact SAU, Spain). Plasma glucose concentrations were determined spectrophotometrically at wavelength 540 nm by using a microplate reader (Biotek HT Sinergy, USA). In addition, plasma insulin concentration was spectrophotometrically quantified at 450 nm by using an ultrasensitive rat insulin enzyme immunoassay commercial kit (Mercodia AB, Sweden) with a microplate reader (Biotek HT Sinergy).

Moreover, plasma concentrations of both glucose and insulin were used to calculate the insulin resistance index (homeostasis model assessment [HOMA]-IR) with the following formula:[23]

$$\mathrm{HOMA-IR} = \text{fasting insulin } (\mu\mathrm{U/mL}) \text{ x } \frac{\text{fasting glucose (mM)}}{22.5}$$

2.7. Lipid metabolism

Plasma cholesterol and triglycerides (TG) were assayed using enzymatic and colorimetric methods with commercial kits (Spinreact S.A/S.A.U, Spain). The concentrations were determined at 450 nm with a spectrophotometer (Biotek HT

175 Sinergy, USA). Results were expressed as mg cholesterol/mL plasma and mg TG/mL
176 plasma.
177
178 *2.8. Histopathological analysis*
179 White adipose tissue and liver was fixed in buffered 10% formalin and
180 embedded in paraffin. Tissues were cut in sections of 5 μm and stained with
181 hematoxylin-eosin (HE) for general analysis. They were studied under a Zeiss Axioskop
182 2 microscope (Zeiss International, USA) equipped with the image analysis software
183 package AxioVision 4.6 (Zeiss International). A qualitative analysis was made in 2 to 4
184 slices of adipose tissue per animal. Besides, adipocyte size was measured counting the
185 number of cells per field under a 20x objective.
186
187 *2.9. Statistical analysis*
188 The results were expressed as mean values ± S.E.M. for a minimum of 6 rats,
189 and were analyzed by Student t test and one or two-way analysis of variance (ANOVA),
190 using the GraphPad Prism 5 software (Graph pad, USA). Differences between the
191 groups were assessed by the Bonferroni post-hoc test. Differences between the means
192 were considered to be significant when $P < 0.05$.
193
194 **3. Results**
195 *3.1. Effects on food and fluid intakes and body composition*
196 Food intake was significantly lower in rats consuming high–fat/high-dextrose
197 diet (MS and MSH) compared to those consuming standard diet (C and CH). No

differences were observed in this parameter in rats consuming hydrolysate (C vs CH and MS vs MSH) (figure 1A). Although there were no differences in fluid intakes between groups before hydrolysate administration (before week 10), MSH rats drunk significantly more fluids than the other groups when they started consuming the hydrolysate (figure 1B). As a consequence, energy intake was significantly higher in MSH rats when they started the hydrolysate consumption, compared to C and MS rats (figure 1C).

As shown in figure 1D, rats consuming high-fat/high-dextrose diet (MS and MSH) showed a significant body weight gain increase than rats consuming standard diet (C and CH). When the hydrolysate consumption started, MSH rats significantly decreased their body weight gain until values similar to C and CH rats. No differences in this parameter were observed in CH vs. C rats.

Regarding to body composition parameters (table 1), at the end of the study abdominal circumference was significantly higher in MS than in C group, and it was significantly lower in MSH rats when compared to MS animals. Body length was also significantly higher in MS rats when compared to C rats, but no differences in this parameter were observed in MSH rats when compared to both C and MS rats.

Relative epididymal adipose tissue weight (table 1) was significantly higher in MS rats compared to C rats. This parameter was significantly reduced in MSH animals when compared to MS group. However, the relative epididymal adipose tissue weight in MSH group did not reach the values of C and CH groups. Although no significant differences were observed, relative liver weight was slightly increased in MS rats

220 compared to C rats (table 1). This parameter was significantly reduced in MSH animals
221 compared to MS animals, reaching control values.
222
223 *3.2. Effects on lipid and glucose metabolism*
224 As shown in table 1, plasma TG levels were significantly higher in high-fat/high-
225 dextrose fed rats (MS and MSH) compared to standard diet fed rats (C and CH). No
226 differences between all groups were observed in plasma cholesterol levels. Regarding
227 HDL cholesterol, no significant differences were shown in MS or MSH animals
228 compared to C animals. However, it is important to note that the rats of CH group
229 presented significantly higher HDL levels than the animals of C group. Similarly, the size
230 of adipocytes was significantly higher in the MS and MSH groups compared to C
231 animals. This hypertrophy significantly decreases in the MSH rats compared to MS
232 animals (figure 2).
233 Plasma glucose levels were significantly higher in MS, compared to C rats (table
234 1). Although there were no significant differences, this parameter was partially
235 reduced in MSH animals when compared to MS group. No differences were observed
236 in CH group compared to C group. Differences in plasma insulin levels were not
237 observed between the experimental groups (table 1). HOMA-IR index was slightly
238 increased in MS group compared to C group and it was slightly decreased in MSH
239 compared to MS rats, but no significant differences were observed in any experimental
240 group (table 1).
241 The presence of tactile allodynia was also evaluated (figure 3). Before
242 hydrolysate consumption, rats consuming high-fat/high-dextrose diet (MS and MSH)

243 presented significantly lower mechanical threshold than rats consuming standard diet
244 (C and CH). This situation was significantly reversed in MSH after the hydrolysate
245 consumption.
246
247 *3.3. Effects on oxidative stress and inflammation*
248 Regarding the plasma antioxidant capacity of the animals (figure 4A), no
249 differences were observed between control group (C) and diet-induced MetS groups
250 (MS and MSH). However, rats of CH group presented significantly higher radical
251 scavenging capacity of plasma compared to C rats. On the other hand, plasma MDA
252 levels (figure 4B) were significantly increased in MS animals when compared to C
253 animals, and this parameter was significantly reduced in MSH, reaching levels similar
254 to C and CH groups. No differences were observed in CH vs. C rats in plasma MDA
255 levels. Liver GSH levels (figure 4C) were increased in MS rats compared to C rats. These
256 values were reduced in MSH group when compared to MS group, and no differences
257 were observed when MSH rats were compared to controls.
258 No significant differences were observed in plasma leptin levels (figure 5A).
259 However, this parameter was slightly increased in MS rats compared to the rest of
260 experimental groups. Plasma adiponectin levels (figure 5B) were significantly increased
261 in MS group compared to C group. MSH rats showed adiponectin values significantly
262 lower than MS group, reaching control-like values. In addition, plasma adiponectin
263 levels were significantly decreased in CH rats compared to control rats.
264
265 **4. Discussion**

In this study Wistar rats were fed a high–fat/high-dextrose diet for 20 weeks to induce MetS. Body weight gain, abdominal obesity, adipose tissue and liver weight were significantly increased in diet-induced MetS rats. Some metabolic parameters, such as plasma glucose, TG, MDA, GSH, and adiponectin also got worse in MS animals compared to C group.

MSH rats attenuated their body weight gain when they started to consume the hydrolysate, in comparison to the MS group, without affecting food intake. Despite the satiating effect that proteins have shown,[24] specially egg white proteins,[25] the consumption of EWH did not produce satiety in the animals of this study. Moreover, Garcés-Rimón *et al.* neither observed this effect in Zucker fatty rats.[18] In addition, fluid intake, and therefore dextrose intake, was increased in MSH rats, thereby increasing energy intake, which enhances the importance of the effects seen on body weight gain in this experimental group. The results found on body composition correspond to those found in body weight gain. The group of MS rats presented abdominal obesity, which is one of the major risk factors in the development of MetS. Visceral fat presents a higher activity in adipokines secretion compared to subcutaneous fat. Some of these adipokines, such as TNF-α or leptin are key roles in the development of hypertension, insulin resistance, and inflammation, related with MetS.[26,27] At the end of the experimental period, those MetS induced animals which consumed EWH, significantly reduced their waist circumference compared to MS rats, decreasing their abdominal fat and therefore, the risk to develop MetS. The reduction in fat mass in MSH rats was also confirmed regarding their adipose tissue weight. Epididymal adipose tissue doubled its size in MS rats compared to C rats, and this increase was partially reduced

289 in MSH animals. Although there were no significant differences, liver weight was also
290 increased in MS animals compared to C rats, which may be related to inflammation
291 and lipid accumulation[28] leading nonalcoholic liver steatosis in this experimental model
292 over time, similar to human metabolic syndrome development. As expected, MSH rats
293 significantly reduced the liver weight, reaching values similar to C group. Garcés-Rimón
294 *et al.* already observed an important prevention in liver steatosis developed in Zucker
295 fatty rats when they consumed this pepsin EWH.[18] All these results suggest a
296 modification in lipid and/or carbohydrates absorption and its metabolism, which leads
297 to a reduction in fat accumulation. Other researchers have also observed similar
298 results using food-derived peptides. Soybean-derived peptides showed to inhibit Fatty
299 Acid Syntase (FAS) activity *in vitro* and to upregulate fatty acid oxidation *in vivo* in mice
300 and diabetic models.[29] Some peptides derived from egg white digested with pepsin
301 have already demonstrated to alter the intestinal lipids uptake by inhibiting their
302 solubilization into micelles.[29]

303 Regarding to lipid metabolism, no differences were observed in cholesterol
304 levels between groups. However, MS rats presented an increase in TG levels, an
305 indicator of abnormal lipid metabolism, which could lead to dyslipidemia, a risk factor
306 of cardiovascular disease.[29] EWH did not seem to have any effect on this alteration
307 developed in MS rats, Garcés-Rimón *et al.* neither observed an effect in TG nor
308 cholesterol levels in Zucker fatty rats,[18] despite the hypocholesterolemic activity that
309 this hydrolysate shown *in vitro*.[17] On the other hand, it was observed an important
310 increase in HDL levels in CH rats, which could imply a protective effect caused by the
311 hydrolysate in healthy animals.

312 Regarding to glucose metabolism, MS rats significantly increased plasma
313 glucose levels, compared to C animals. No changes in plasma insulin levels were
314 observed between different experimental groups. HOMA-IR index was slightly
315 increased in MS group which could indicate an early state of insulin resistance. In
316 addition, MS rats presented tactile allodynia, which is a symptom of sensory
317 neuropathy, and appears at early stages of diabetes.[3] This alteration is also described
318 in MetS situation in rats fed high-energy/high-sucrose diets.[21] According to these
319 results, we conclude that MS rats developed an early insulin resistance, with an
320 important sensory neuropathy. This pre-diabetes stage is reverted after EWH
321 consumption. MSH rats presented slightly reduced glycaemia compared to MS animals,
322 and the diabetic neuropathy was also reversed when the animals started to consume
323 the EWH. There are studies which describe hypoglycemic food hydrolysates and
324 peptides which act as dipeptidyl peptidase IV (DPP-IV) inhibitors both *in vitro* and *in*
325 *vivo*[30] from different sources, including egg white.[31] The EWH used in this study had
326 previously demonstrated a DPP-IV inhibitory activity *in vitro*[17] as well as lowering
327 plasma insulin properties in Zucker fatty rats (data not published); in this study we
328 have not observed a reduction in plasma insulin levels, due to this model of MetS did
329 not present increased insulin plasma levels. However, we could observe an important
330 hypoglycemic activity by reduction in plasma glucose levels, which could not be
331 observed in Zucker fatty rats due to the normoglycemic characteristics of this genetic
332 MetS model. Therefore, these results obtained in glucose metabolism complement
333 those obtained by our research group (data not published) and suggest that this EWH
334 could be used to control alterations of glucose metabolism associated to MetS.

It is well known the key role of oxidative stress in the development and maintenance of the MetS and related complications.[32] Antioxidant foods have been suggested as a strategy to reduce or ameliorate this pathology or some of its complications.[33] Moreover, oxidative stress has been also related with diabetic neuropathy, being a possible target in pharmaceutical intervention, especially using peptide-based antioxidants.[34] By this way, an improvement in the oxidative stress could help to ameliorate the most of complications related to MetS. In this study, high-fat/high-dextrose fed rats (MS and MSH groups) did not shown differences in their plasma antioxidant capacity compared to C animals. However, there was a significant improvement in the plasma ORAC values of CH rats compared to C group. This result could be related to the increase in HDL cholesterol levels, also observed in this group. HDL is considered an important plasma antioxidant defense system, which acts by preventing the oxidation of LDL.[35] It suggests that the consumption of EWH can produce an increase of the antioxidant defense in healthy situations to provide high protection in the presence of oxidative stress related diseases.

MDA is used as a biomarker of lipid peroxidation. Its determination in plasma is one of the most useful methods to predict the oxidative stress levels.[36] Plasma MDA levels were significantly increased in MS rats, so we can confirm the presence of increased oxidative stress in this animal model. This parameter was, on the contrary, significantly decreased in plasma of MSH animals at the end of the study. These results agree with those obtained by Garcés-Rimón et al. in Zucker fatty rats[18] and confirm that the EWH studied could reduce the oxidative stress associated to MetS conditions. It is worth emphasizing that EWH exhibited antioxidant properties and hydroxyl radical

358 scavenging capacity in previous *in vitro* studies.[17] In addition, we found an increase in
359 liver GSH levels in MS animals. This increase has been also observed in high fructose-
360 fed Sprague Dawley rats.[37] It has been suggested that increased GSH levels in liver may
361 be an early tissue defense mechanism against oxidative stress. This alteration in liver
362 GSH levels was attenuated in MSH rats, which indicates the presence of a
363 compensatory mechanism on oxidative stress situation after consumption of the EWH.
364 These results suggest that the intake of EWH could have a protective role not only in
365 subjects with MetS and/or oxidative damage conditions but also in non-pathological
366 situations, keeping a prepared antioxidant defense that can act rapidly in punctual
367 oxidative stress situations.

368 The relationship between obesity and inflammation has been extensively
369 studied. White adipose tissue has been recognized as endocrine organ which mediates
370 the development of MetS in obese subjects due to secretion of adipokines.[38] Leptin,
371 one of the most studied adipokine, plays an important role in the regulation of satiety
372 and food intake. Obese patients typically present high circulating leptin levels due to
373 the development of leptin resistance;[26] it has been also shown that leptin can directly
374 modulate the immune system, acting as a pro-inflammatory factor.[38] No significant
375 differences were observed in circulating leptin levels between different experimental
376 groups. MS rats showed a tendency to increase their plasma circulating leptin levels
377 but, however, this increase was not observed in MSH obese animals after consumption
378 of EWH. This result suggests a leptin sensitization caused by the EWH consumption
379 that, together with a less fat mass in these animals, could lead to a decreased leptin
380 levels in plasma. It would also mean a reduction of the inflammation signaling in MSH

animals compared to MS. On the other hand, adiponectin is recognized as an anti-inflammatory adipokine with protective effects against MetS.[26] However, MS group increased circulating adiponectin levels in plasma. This unexpected adiponectin increase has been also observed in palatable diet-fed C57BL/6 mice[39] and in Zucker Fatty rats.[18] Garcés-Rimón *et al.* associated high levels of this adipokine with adiponectin resistance.[18] Actually, it has been also observed less expression of adiponectin receptors in hyperinsulinemic and hyperglycemic states.[40] MSH rats showed adiponectin levels similar to C rats, and much lower than MS animals. It suggests that the consumption of EWH reverted or protected against adiponectin resistance developed in MS group during the study.

In conclusion, the present study showed that the oral administration of a pepsin EWH could be used as a functional ingredient to improve some complications associated to MetS induced by unhealthy diets. More research is required to go in depth the mechanisms and molecular pathways involved in their beneficial effects, and human clinical studies are necessary to consider its use to prevent or treat these complications in MetS patients.

Conflict of interest

All authors certify that there is no conflict of interests in this study.

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528

529 **Figure captions**

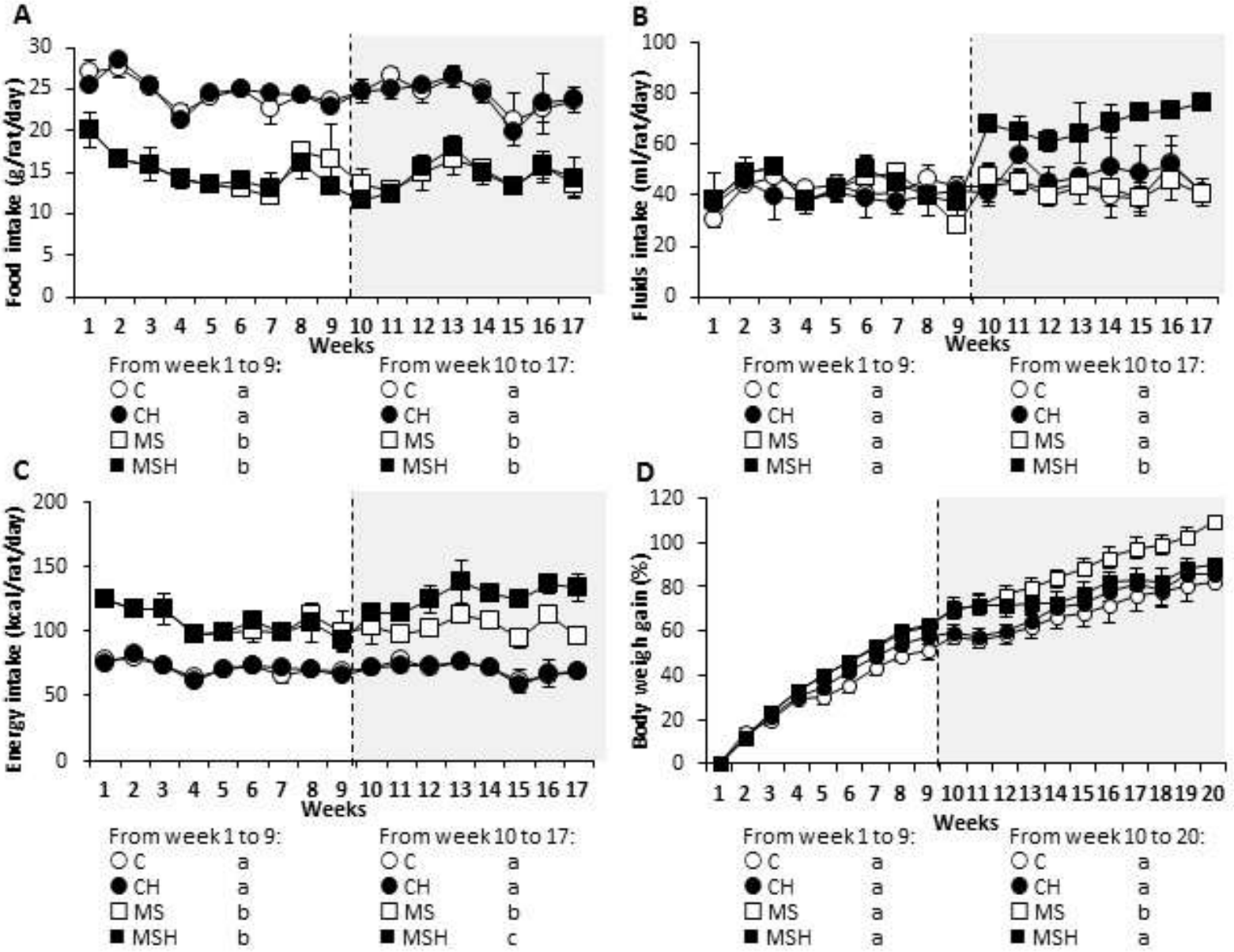
530 **Figure 1.** Food intake (A), fluids intake (B), energy intake (C) and body weight gain
531 during the study. Experimental groups: standard-fed rats (○), standard-fed rats
532 receiving 1g/kg/day of Egg White Hydrolysate (EWH) (●), diet induced obese rats (□)
533 and diet induced obese rats receiving 1g/kg/day of EWH (■). The rats were treated
534 with the pepsin EWH since week 10^{th} until the end of the study. Values are means ±
535 SEM (n=7-10). Different letters mean that values are significantly different ($p<0.05$)
536 among groups.

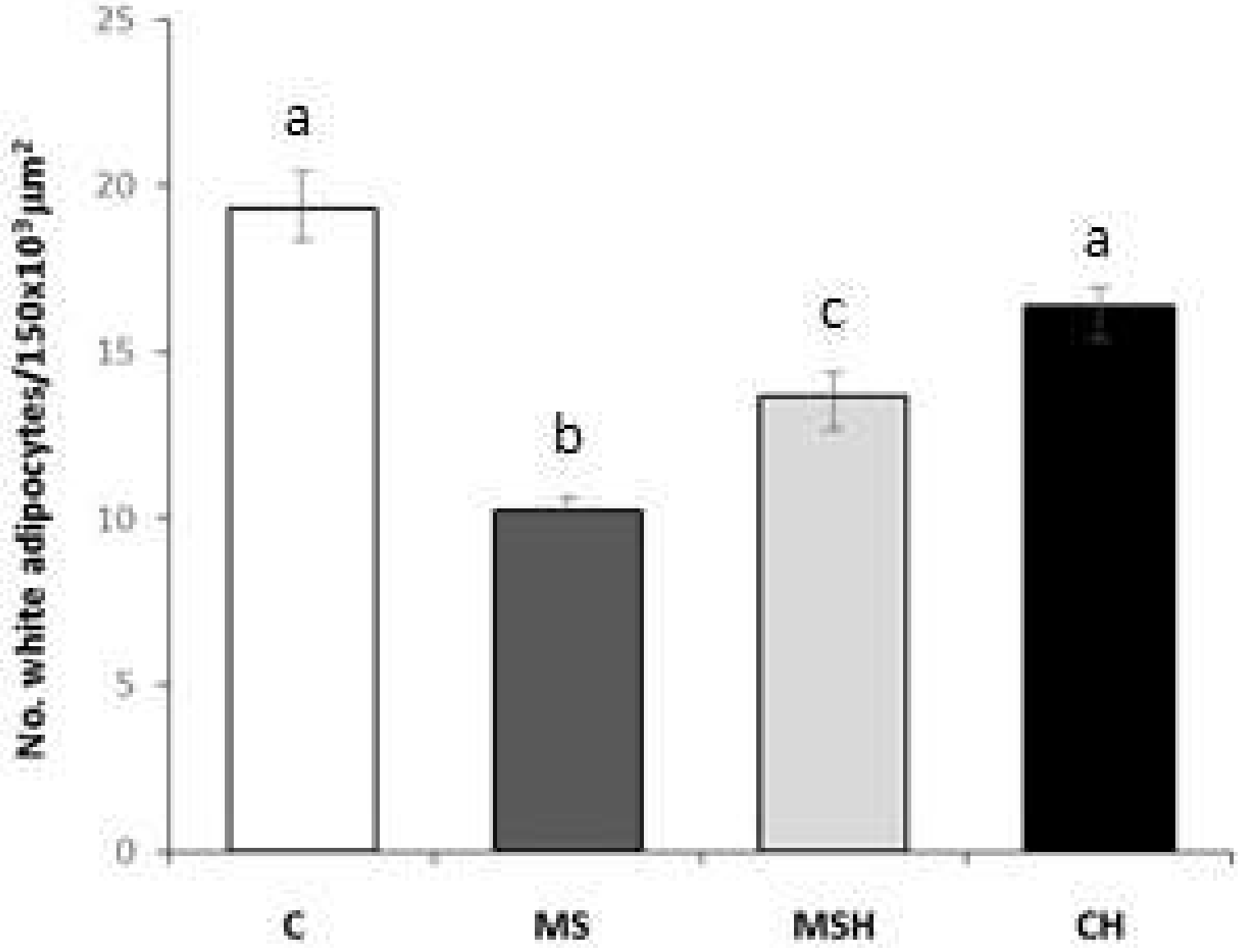
537 **Figure 2.** Number of white adipocytes per 20x field in histological sections of
538 epididymal adipose tissue. Experimental groups: Standard-fed rats (C), standard-fed
539 rats receiving 1g/kg/day of Egg White Hydrolysate (EWH) (CH), diet induced obese rats
540 (MS) and diet induced obese rats receiving 1g/kg/day of EWH (MSH). Animals were
541 treated with the pepsin EWH since week 10^{th} until the end of the study (week 20^{th}).
542 Values are means ±SEM (n=7-10). Different letters mean that values are significantly
543 different ($p<0.05$) among groups.

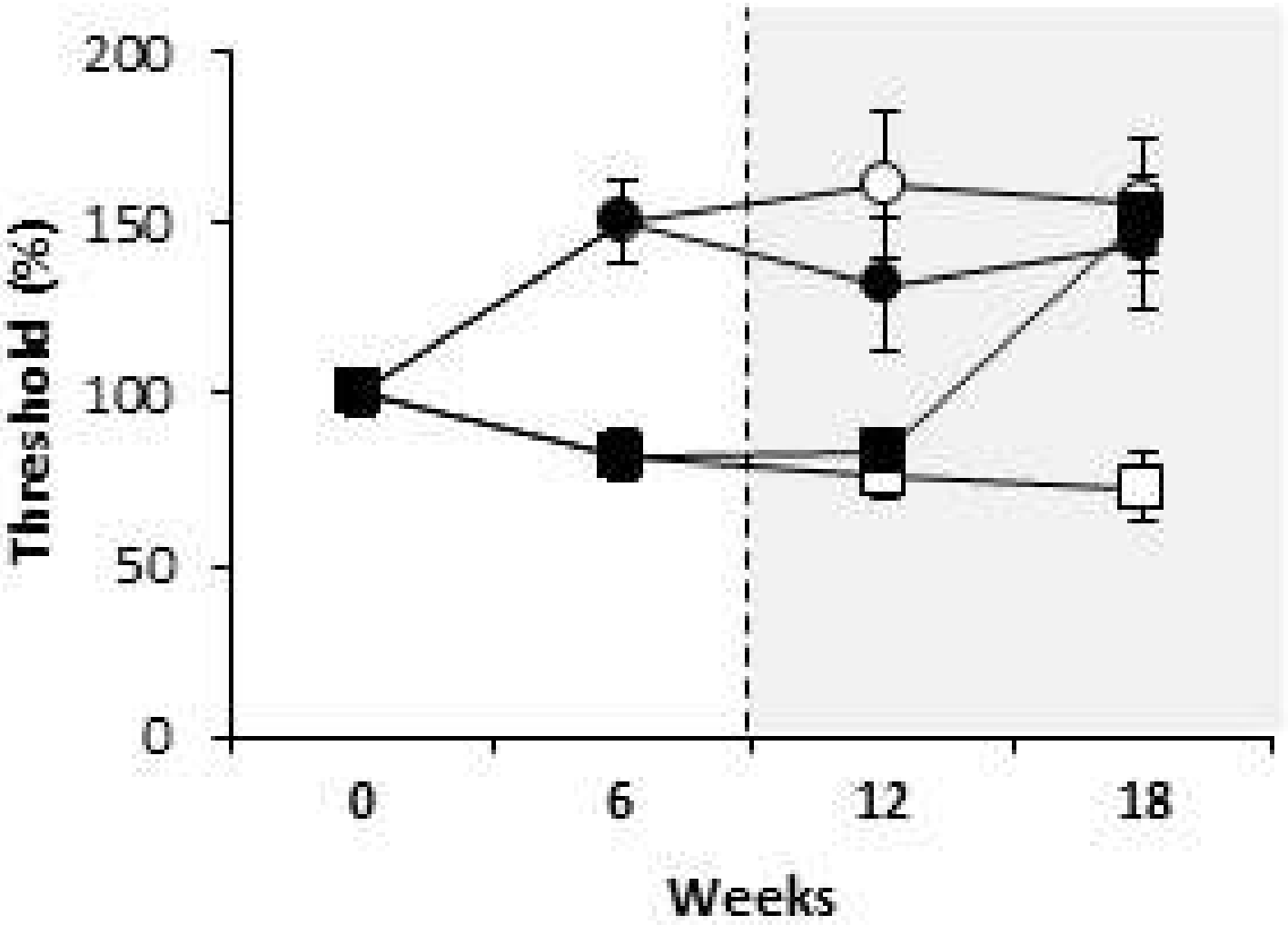
544 **Figure 3.** Mechanical sensitivity evolution during the study. Experimental groups:
545 standard-fed rats (○), standard-fed rats receiving 1g/kg/day of Egg White Hydrolysate
546 (EWH) (●), diet induced obese rats (□) and diet induced obese rats receiving
547 1g/kg/day of EWH (■). The rats were treated with the pepsin EWH since week 10^{th}
548 until the end of the study. Values are means ±SEM (n=7-10). Different letters mean
549 that values are significantly different ($p<0.05$) among groups.

550 **Figure 4.** Plasma antioxidant capacity (A), plasma malondialdehyde (MDA) levels (B)
551 and liver reduced glutathione levels (C) at the end of the study in the different groups:

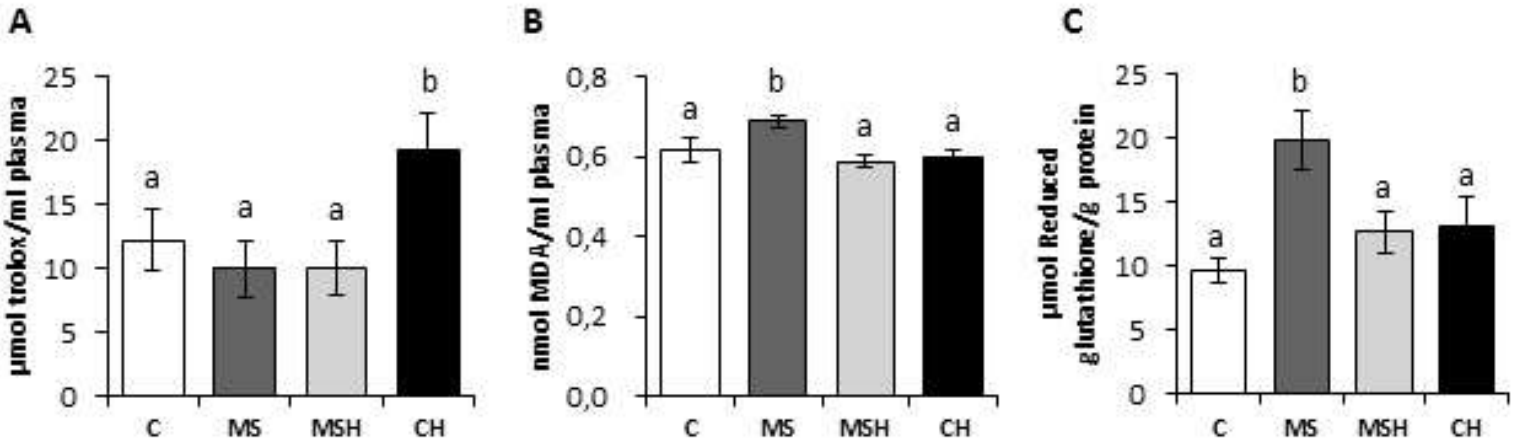
552 standard-fed rats (C), standard-fed rats receiving 1g/kg/day of Egg White Hydrolysate
553 (EWH) (CH), diet induced obese rats (MS) and diet induced obese rats receiving
554 1g/kg/day of EWH (MSH). The rats were treated with the pepsin EWH since week 10^{th}
555 until the end of the study (week 20^{th}). Values are means ±SEM (n=7-10). Different
556 letters mean that values are significantly different ($p<0.05$) among groups.
557 **Figure 5.** Plasma leptin (A) and plasma adiponectin (B) levels at the end of the study in
558 the different groups: standard-fed rats (C), standard-fed rats receiving 1g/kg/day of
559 Egg White Hydrolysate (EWH) (CH), diet induced obese rats (MS) and diet induced
560 obese rats receiving 1g/kg/day of EWH (MSH). The rats were treated with the pepsin
561 EWH since week 10^{th} until the end of the study (week 20^{th}). Values are means ±SEM
562 (n=7-10). Different letters mean that values are significantly different ($p<0.05$) among
563 groups.
564
565 **Table 1**: Results in body composition, tissue and organs weights, and lipid and glucose
566 metabolism at the end of the study. Experimental groups: standard-fed rats (C),
567 standard-fed rats receiving 1g/kg/day of Egg White Hydrolysate (EWH) (CH), diet
568 induced obese rats (MS) and diet induced obese rats receiving 1g/kg/day of EWH
569 (MSH). The rats were treated with the pepsin EWH since week 10th until the end of
570 the study (week 20th). Values are means ± SEM (n= 7-10). Different superscript letters
571 mean that values are significantly different ($p<0.05$) among groups.

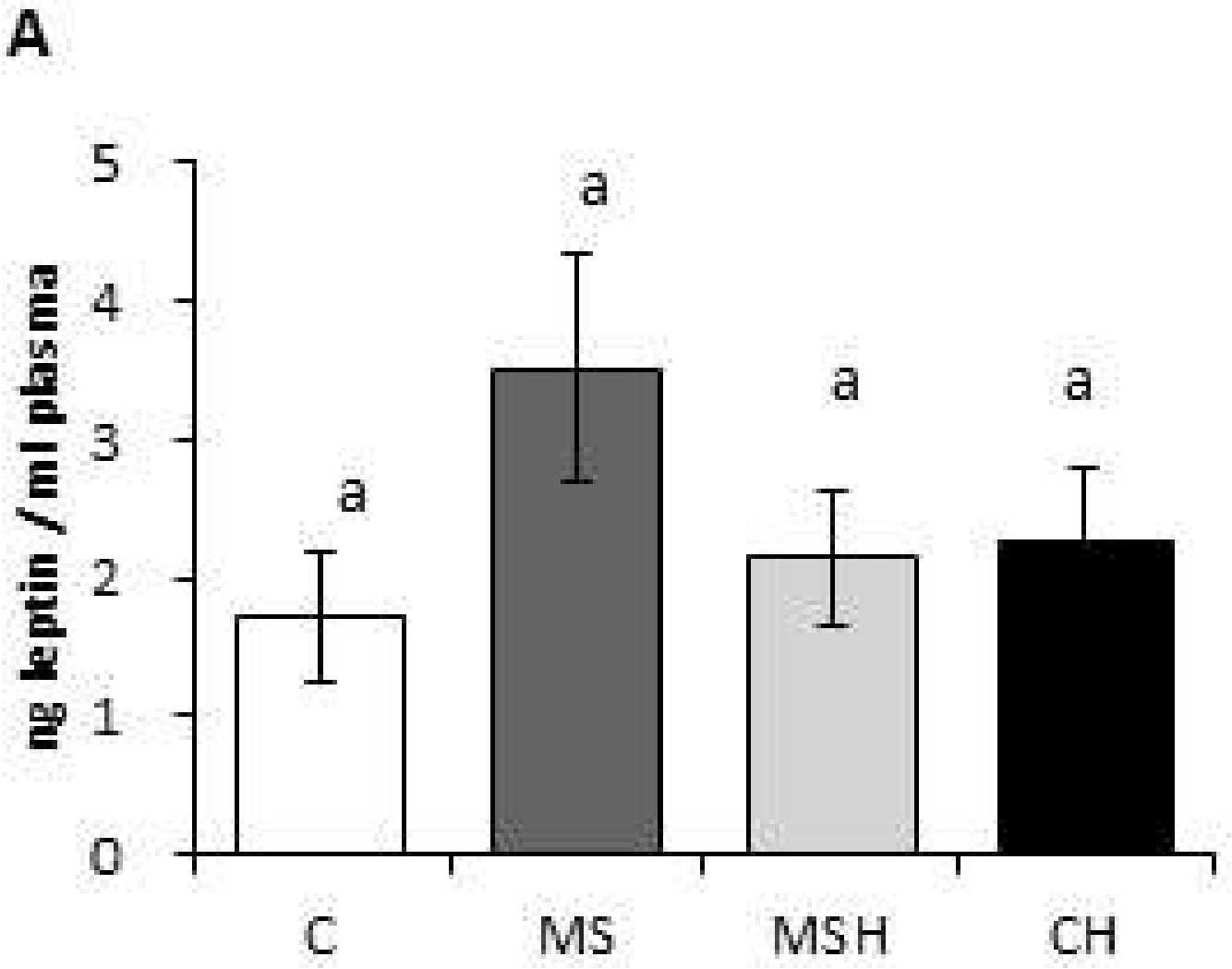

A
Food intake (g/rat/day)
Weeks
From week 1 to 9:
C a
CH a
MS b
MSH b
From week 10 to 17:
C a
CH a
MS b
MSH b
B
Fluids intake (ml/rat/day)
Weeks
From week 1 to 9:
C a
CH a
MS a
MSH a
From week 10 to 17:
C a
CH a
MS a
MSH b
C
Energy intake (kcal/rat/day)
Weeks
From week 1 to 9:
C a
CH a
MS b
MSH b
From week 10 to 17:
C a
CH a
MS b
MSH c
D
Body weigh gain (%)
Weeks
From week 1 to 9:
C a
CH a
MS a
MSH a
From week 10 to 20:
C a
CH a
MS b
MSH a

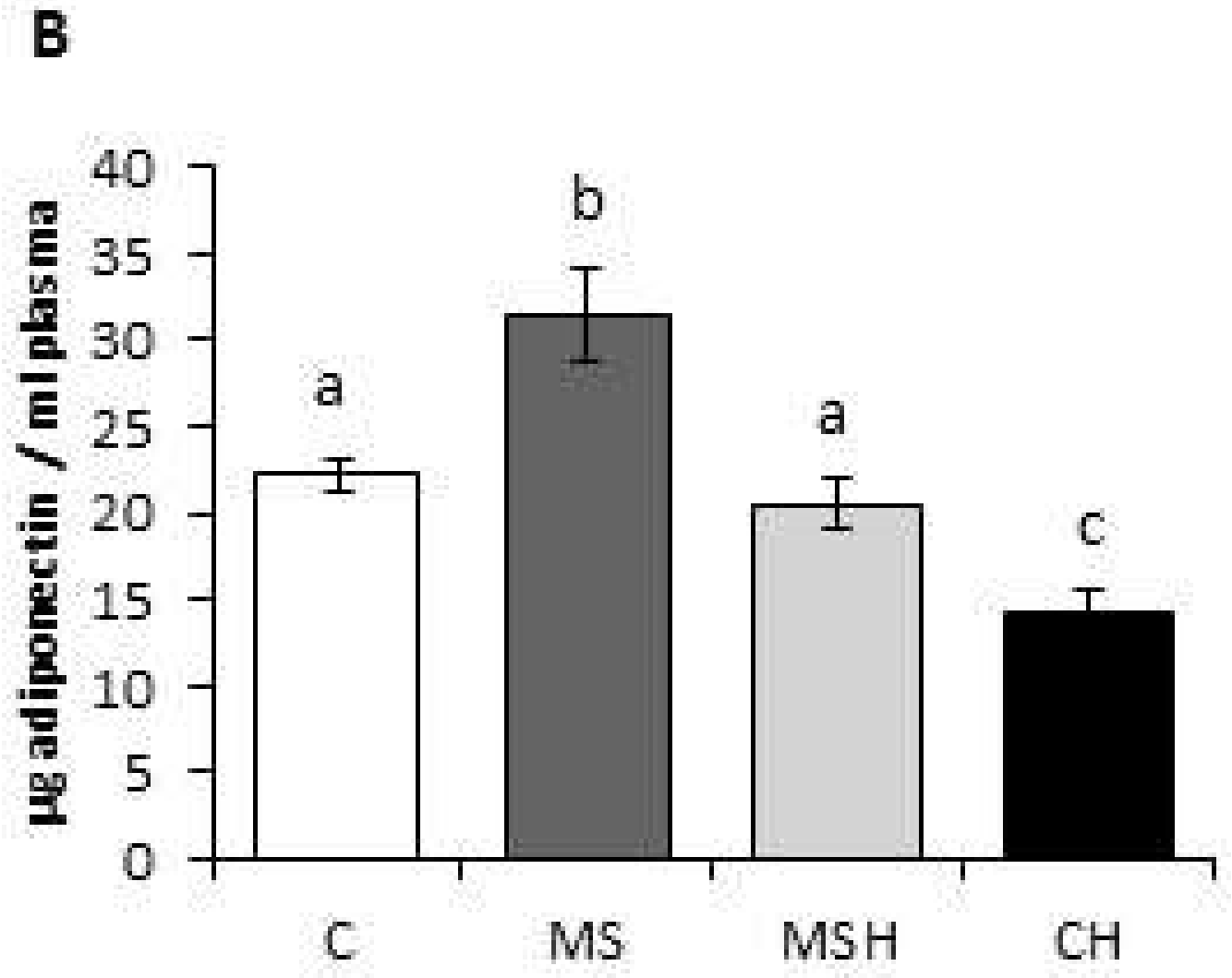

No. white adipocytes/1.50x10³µm²
25
20
15
10
5
0
a
b
c
a
C
MS
MSH
CH



From week 1 to 9:		From week 10 to 20:	
○ C	a	○ C	a
● CH	a	● CH	a
□ MS	b	□ MS	b
■ MSH	b	■ MSH	a


A
µmol trolox/ml plasma
25
20
15
10
5
0
a
a
a
b
C
MS
MSH
CH
B
nmol MDA/ml plasma
0,8
0,6
0,4
0,2
0,0
a
b
a
a
C
MS
MSH
CH
C
µmol Reduced glutathione/g protein
25
20
15
10
5
0
a
b
a
a
C
MS
MSH
CH


A
ng leptin / ml plasma
0
1
2
3
4
5
a
a
a
a
C
MS
MSH
CH


B
µg adiponectin / ml plasma
0
5
10
15
20
25
30
35
40
a
b
a
c
C
MS
MSH
CH

Variable	C	MS	MSH	CH
Body composition				
Abdominal circumference, cm (n=7-10)	22,01 ± 0,34^{a}	25,51 ± 0,44^{b}	23,77 ± 1,10^{c}	21,96 ± 0,31^{a}
Body length, cm (n=7-10)	24,94 ± 0,24^{a}	25,65 ± 0,69^{b}	25,25 ± 1,32^{ab}	26,11 ± 1,21^{b}
BMI (n=7-10)	0,87 ± 0,03^{a,b}	0,92 ± 0,01^{b}	0,89 ± 0,02^{a,b}	0,80 ± 0,04^{a}
Tissue and organs wet weights, g/cm tibial length (n=7-10)				
Epididymal adipose tissue	3,65 ± 0,24^{a}	6,10 ± 0,47^{b}	5,03 ± 0,16^{c}	3,52 ± 0,30^{a}
Liver	2,89 ± 0,09^{a,b}	3,04 ± 0,1^{b}	2,74 ± 0,09^{a}	3,05 ± 0,11^{b}
Lipid metabolism				
Triglycerides, mg/dl (n=7-10)	38,76 ± 2,07^{a}	57,97 ± 7,15^{b}	65,90 ± 3,67^{b}	43,97 ± 3,03^{a}
Cholesterol, mg/dl (n=7-10)	53,45 ± 4,00^{a}	51,18 ± 2,84^{a}	56,49 ± 2,72^{a}	56,63 ± 3,73^{a}
HDL, mg/dl (n=7-10)	8,92 ± 1,09^{a}	6,63 ± 1,64^{a}	6,55 ± 1,42^{a}	13,06 ± 0,38^{b}
Glucose metabolism				
Glucose, mg/dl (n=7-10)	226,4 ± 18,9^{a}	327,7 ± 28,3^{b}	277,4 ± 16,1^{a,b}	249,6 ± 25,7^{a,b}
Insulin, µmol/ml (n=7-10)	4,15 ± 1,27^{a}	4,44 ± 0,65^{a}	4,36 ± 0,98^{a}	4,02 ± 0,76^{a}
HOMA-IR (n=7-10)	0,025 ± 0,009^{a}	0,036 ± 0,015^{a}	0,028 ± 0,008^{a}	0,025 ± 0,013^{a}