AN ABSTRACT FOR THE THESIS OF

Betty J. Herring for the Master of Science Degree

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Title:

Effects of long-term exposure to $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$ in Wistar rats fed normal and high-fat diets.

Abstract approved:

The idea that chromium(III) is an essential element has been debated for over 50 years. Just as the element's essentiality is debated, so are the effects of chromium(III) supplementation, particularly in healthy subjects. Chromium(III) is widely used as a nutritional supplement for weight loss, building of lean muscle mass, and improving glucose and lipid metabolism. In contrast to most chromium supplements, which provide the body with a source of chromium(III), chromodulin is thought to be a biologically active chromium(III)-containing compound in the body that, when bound to insulin receptors, helps the receptors remain active longer. $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$, or Cr3, is a chromodulin biomimetic and is the chromium compound used in this study. The current study evaluates the effects of long-term supplementation on body mass and glucose metabolism in Wistar rats on traditional and cafeteria-style (high fat, high carbohydrate) diets. Male Wistar rats were randomly assigned to one of four treatment groups: 1) control diet (milled Harlan Teklad LM-485 rodent diet), 2) control diet + 1 mg Cr3/kg body mass/day, 3) a cafeteria-style (CAF) diet (high fat, high carbohydrate), or 4) CAF diet + 1 mg Cr3/kg/day. Cr3 supplementation had no effect on blood glucose levels or responses to glucose and insulin challenges. Rats consuming the CAF + Cr3 diet

tended to have a significantly higher body mass than rats consuming the CAF diet, but necropsy results showed no difference in visceral fat or body wall thickness between groups. These data suggest that long-term Cr3 supplementation does not significantly affect body mass in rats consuming a normal diet or metabolic responses in rats consuming either diet. Further study is needed to elucidate the mechanism(s) behind the effect of Cr3 on body mass in rats consuming a high-fat, high-carbohydrate diet.

EFFECTS OF LONG-TERM EXPOSURE TO $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$ IN WISTAR RATS FED NORMAL AND HIGH-FAT DIETS

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PREFACE

This paper was prepared in the style of Biological Trace Element Research to which it will be submitted.

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INTRODUCTION

The idea that chromium(III) is an essential element for proper glucose and fat metabolism in mammals has been debated for more than 50 years. Initially, Schwarz and Mertz [1, 2], proposed that a "glucose tolerance factor" (GTF) was naturally present in the body and that this compound contained chromium [3, 4]. This belief was in part a result of studies in which rats on chromium-deficient diets developed impaired glucose tolerance. When chromium was given to deficient rats by stomach tube, the impairment was reversed [1].

Case studies of humans on total parenteral nutrition (TPN) who developed glucose intolerance and other problems such as neuropathy and encephalopathy have also been cited as evidence of the essentiality of chromium(III) because the addition of chromium improved their symptoms [4, 5]. However, the validity of such an assumption has been questioned. In a review of the case studies, Stearns [3] notes that among the five cases, the time on TPN varied from five months to 13 years. Chromium is already present in TPN solutions as a contaminant, at levels of 4.5 µg/L to over 50 µg/L [3]. The reported chromium concentrations in the TPN solutions ranged from 2µg- 6µg per day but did not take into account chromium levels already present as contamination in the TPN solutions. Chromium absorption in the gut varies widely, depending on the type of ligand to which it is attached, but the chromium in TPN solution is "absorbed" completely as it is given intravenously [3]. So, persons on TPN solutions are not necessarily chromium-deficient, once contamination and absorption are taken into account. Additionally, not all patients

shared the same combination of symptoms, while chromium-deficient patients were treated with varying amounts of chromium once symptoms developed, and glucose intolerance did not improve with addition of chromium in one of the cases [3].

The National Academy of Sciences set the adequate dietary chromium intake at 25 µg/day for women and 35 µg/day for men [6]. By this standard, almost all Americans have a chromium-sufficient diet [4, 7]. Chromium deficiency is difficult to diagnose, because at this time no clinical method for determining whether an individual is chromium deficient has been established [3]. Dietary carbohydrate stress increases urinary chromium loss, so a high-carbohydrate diet has been used to create chromium deficiency in animals for the purpose of studying the effects of deficiency and subsequent supplementation [4]. Many parameters have to be considered when designing an experiment involving chromium deficiency. In addition to controlling diets, one must prevent access to stainless steel and perhaps apply additional dietary stress to increase an animal's chromium loss [4]. Metal cage components, usually made of stainless steel, contain chromium, and traditional rodent bedding contains metal ions as well. Use of these items could skew the results in this type of study [7]. Studies claiming chromium's essentiality are often flawed because they do not take these types of housing factors into consideration. Also, some studies using "low-chromium" diets do not consider the size of the test animal or the amount of food the animal will consume. One such study used a "low-chromium" diet that, when the above factors were figured in, contained a dosage of ten times what humans take in per kg body mass [7].

For an inorganic element to be considered essential, it must meet certain requirements. It must be naturally present in the body, have a definite biological

function, and deficiency of the element results in some impairment, which can be reversed when the element is returned to the diet [3]. Chromium(III) is naturally present in the body. However, a study using a purified (chromium-free) diet and metal-free housing did not produce any impairment in chromium-deficient rats [7]. So, by this definition, chromium does not appear to meet all of the requirements for classification as an essential element.

Just as the essentiality of chromium(III) has been heavily debated, the effects of nutritional chromium(III) supplementation have been debated, particularly in healthy subjects [8, 9]. Chromium(III) is widely used as a nutritional supplement for weight loss, building of lean muscle mass, and improving glucose and lipid metabolism, despite a lack of conclusive support of these claims in human subjects [10, 11, 12, 13, 14]. Dietary chromium is absorbed with 0.4-2% efficiency [15], so coupling chromium with a suitable ligand is desirable to increase bioavailability. Commercially available chromium supplements (particularly chromium picolinate) are very popular as "natural" complements to traditional pharmaceutical treatments for type 2 diabetics and metabolic syndrome, but the results are inconclusive as to whether or not supplementation is useful [9, 16, 17, 18, 19].

In contrast to other chromium supplements, which merely provide the body with a source of bioavailable chromium(III), low-molecular-weight chromium-binding substance (LMWCr), or chromodulin, may be a biologically active chromium(III)-containing compound in the body. This peptide clears chromium from the tissue for expulsion in the urine and it has been proposed to stabilize the insulin receptor in its

active conformation [20]. This hypothesis is supported by *in vitro* studies, but this proposed mechanism for increasing insulin sensitivity has not been established *in vivo*.

$[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$, or Cr3, is a chromodulin biomimetic. Cr3 is more water-soluble than chromium nicotinate and chromium picolinate (the two most popular chromium supplements) and does not break down in the GI tract like chromium chloride and chromium nicotinate [21]. Cr3 has been shown to decrease fasting plasma triglycerides and cholesterol in healthy and diabetic rats, as well as decreasing plasma insulin concentrations and increasing insulin sensitivity in diabetic rats [21].

To date, most of the available literature reports on the results of short-term studies exploring the effects of chromium supplementation in rats, but few have looked at the effect of long-term chromium supplementation. No reports to date have appeared on the effects of long-term supplementation with Cr3. Thus, the current study evaluates the effects of long-term supplementation on body mass and glucose metabolism in Wistar rats on traditional and cafeteria-style (high fat, high carbohydrate) diets.

MATERIALS AND METHODS

Animals and Exposure

Forty-eight male Wistar rats, obtained from Charles River Breeding Laboratories (Portage, MI), were housed in a USDA-approved animal facility in rooms maintained at 25 ± 2 °C at 50 % to 70 % humidity and a 12 hour photoperiod. After a two week acclimation period, each rat was uniquely identified by ear punch and randomly assigned to one of four treatment groups: 1) control diet (milled Harlan Teklad LM-485 rodent diet) (n = 11), 2) control diet + 1 mg Cr3/kg body mass/day (n = 12), 3) a cafeteria-style (CAF) diet (33 % powdered Harlan Teklad LM 485 rodent chow, 33% sweetened condensed milk, 27 % water, and 7 % granulated sucrose) [22] (n = 12), or 4) CAF diet + 1 mg Cr3/kg/day (n = 12). Rats were individually housed in shoe-box type cages with recycled bedding and were allowed to consume the appropriate diet and water *ad libitum*.

Cr3 was synthesized according to the methods of Earnshaw et al. [23] and generously provided by the Vincent laboratory at The University of Alabama. The authenticity of the Cr3 was established by high resolution electron impact mass spectrometry [24]. Milled rodent diet was purchased from Harlan Teklad (Madison, WI). Cr3 was added to the appropriate diets in sufficient quantities to achieve the appropriate concentration of the test compound. All calculations were based on data from a previous study which indicated that male Wistar rats consume an average of 33 g diet/day. Extensive stability studies indicate that chromium test compounds are extremely stable and no degradation in the diet would be expected [25].

Because the purpose of this study was to determine the effects of pharmaceutical levels of chromium, no special measures were taken to prevent exposure of the rats to small amounts of chromium that may be introduced into the diet through methods of feed preparation or from the cage hardware. The diet purchased, Teklad LM-485 (7012), contained added chromium in the form of chromium potassium sulfate (0.48 mg/kg of diet).

Data Collection

Body mass was measured bi-weekly. Blood samples were obtained monthly from the lateral saphenous vein following a 12 hour fast. Samples were centrifuged, and plasma was removed and frozen at -80 °C for later analysis of insulin content by enzyme-linked immunosorbant assay (ELISA). Blood glucose was also measured at the time of collection using a One-Touch Ultra™ glucose meter. Data collection continued for 15 months, during which time 3 glucose challenges and 2 insulin challenges were also administered. Glucose was administered intraperitoneally at a dosage of 2 g/kg body mass, and blood glucose levels were measured prior to injection and 30, 60, 90, and 120 minutes after injection. Bovine zinc insulin (5 units/kg body mass) was administered subcutaneously, and blood glucose levels were measured as in the glucose challenges.

After 15 months, rats were euthanized and necropsied. Final body mass, body wall thickness, organ masses (liver, kidneys, lungs, and heart), and visceral fat mass were measured. Data from animals that died prior to 15 months were not included in the analyses, but those animals were necropsied and measured.

Statistical Methods

Data means were analyzed by one-way ANOVA using SPSS (SPSS, Inc., Chicago, IL) followed by a Fisher's LSD post-hoc multiple comparisons test to determine specific significant differences ($p \leq 0.05$).

RESULTS AND DISCUSSION

There was no significant difference ($p > 0.05$) among fasting blood glucose levels.(Figure 1). Results of glucose challenges, administered at months 4 and 14, are shown in Figures 2 and 3. There were differences in the rats' responses to glucose challenges, but area under the curve analysis (Figures 4 and 5) shows that the differences were not consistent or biologically meaningful (Table 1). Thus, it can be concluded that rats on either diet with or without Cr3 supplementation could handle an increase in glucose load without an appreciable increase in blood glucose levels. Cr3 exposure did not affect the rats' responses to the insulin tolerance tests administered at month 10 and month 14 of the study, as shown in the blood glucose levels (Figures 6 and 7) and the respective AUCs (Figures 8 and 9). Thus, all of the rats were able to manage their blood glucose levels after the administration of exogenous insulin equally well. Conclusive data about the effect of diet or Cr3 supplementation on fasting plasma insulin levels are not available. Fasting plasma insulin levels were measured by ELISA, but for unknown reasons, many of the samples did not yield usable data. Those samples that were readable showed no significant difference in insulin levels between the groups (data not shown).

Rats consuming the CAF + Cr3 diet tended to have a significantly ($p \leq 0.05$) higher body mass than rats consuming the CAF diet (Figure 10). As chromium supplements are purported to reduce fat and increase muscle mass, one would expect the increase in body mass to be attributed to changes in these parameters. However, an examination of the visceral measurements (Table 2) reveals that there were no statistically significant increases in body wall thickness (a measure of muscle tissue) or reduction in visceral fat. In addition, if Cr3 supplementation had increased lean muscle

mass and decreased visceral fat mass, this effect should have been paralleled in the rats consuming the control diet. However, these parameters are nearly identical between these groups. Rats consuming the CAF diet, with or without Cr3 supplementation, had significantly higher body mass than rats consuming either of the control diets (Figure 10). This difference appears to stem primarily from an increase in visceral fat, and not from an increase in lean muscle tissue (Table 2).

Cr3 is a novel chromium supplement that is a biomimetic of chromodulin. Chromodulin, a peptide that clears chromium from the tissue for expulsion in the urine, has also been proposed to be able to stabilize insulin receptor in its active conformation [20]. In this proposed mechanism, when plasma insulin levels rise, insulin binds to its receptors, and chromium is moved from the blood (where it is stored as its transferrin complex) into target cells (primarily insulin-sensitive skeletal muscle cells, adipocytes, and hepatocytes). Chromium binding to apochromodulin forms holochromodulin, which in turn binds to the intracellular side of the insulin receptor. This binding allows the receptor to maintain its active conformation longer [20]. This mechanism, however, has not been supported to date by *in vivo* studies. A short-term study in 2005 study by Clodfelder et al. [26] showed that administration of Cr3 by gavage significantly lowered fasting blood insulin levels as well as blood glucose concentrations 2 hours after glucose challenges in Sprague-Dawley rats. This study's results did not reflect the same effect, possibly due to strain differences or differences in absorption (bolus dosing versus administration in feed).

The results of this study indicate that long-term supplementation with Cr3, a chromium compound that is biologically active *in vitro*, is not beneficial to healthy or

obese rats with regard to glucose metabolism or body composition. Other studies with various chromium compounds have yielded similar results, although supplementation with these compounds was not as long in duration. A recent study by Di Bona et al. [7] showed that chromium supplementation [in the form of $KCr(SO_4)_2 \cdot 12H_2O$] failed to lead to an increase in lean muscle or a decrease in body mass in healthy rats. Other studies utilizing other forms of chromium(III) have reported similar results (for review, see Vincent [4]). Chromium treatment has no effect on body mass or composition in individuals with type 2 diabetes [27] or non-diabetic adults with metabolic syndrome [28]. Nutritionally-relevant doses of chromium have no effect on glucose or insulin levels [11], ability to handle glucose and insulin challenges [7], plasma cholesterol [5], or blood lipid levels [4] in either healthy humans or in animals. Pharmacological doses of chromium(III) have been shown to increase insulin sensitivity in healthy lean rats [7], but nutritional chromium supplementation apparently has no observable benefit for healthy individuals on a chromium-sufficient diet [4, 14]. The dosage in this study was pharmacological, 1 mg Cr3/kg body mass/day. One mg of Cr3 contains 0.217 mg Cr, so an average (70 kg) man receiving 1 mg Cr3/kg body mass/day would be consuming 15.19 mg Cr/day. Typically, chromium nutritional supplements such as chromium picolinate are available as 200 – 800 µg pills and are absorbed much less efficiently than Cr3.

Studies in rodent models of type 1 and type 2 diabetes almost uniformly observe beneficial effects from chromium supplementation, whereas studies on the effects on diabetic human subjects generally observe no effect. Isolated cases of TPN patients exhibiting impaired glucose tolerance were given pharmacological doses of chromium in

the form of chromium chloride and saw those symptoms improve [29, 30, 31]. A review of human randomized controlled trials suggests that pharmacological chromium supplementation improved fasting glucose and glucose metabolism (as evidenced by lower glycosylated hemoglobin levels) in diabetics, but not non-diabetics with glucose intolerance [14]. Cefalu et al. [5] found that pharmacological doses of chromium picolinate increased insulin sensitivity in hyperinsulinemic obese rats but not in lean controls. However, other studies have not shown chromium supplementation to improve overall glucose metabolism in individuals with metabolic syndrome [28], and a review of chromium studies published in 2001 found that chromium supplementation may have little or no effect on diabetic subjects [4].

The data obtained in this study strongly suggest that long-term Cr3 supplementation does not significantly affect body mass or metabolic responses to glucose or insulin in rats consuming a normal diet or a high-fat, high-carbohydrate cafeteria-style diet. These results, along with the results of many shorter-duration studies [4, 7, 27, 28] clearly support the idea that chromium is not an essential element for proper glucose metabolism. The effects of long-term supplementation on lipid parameters such as total cholesterol, low-density lipoprotein, and high-density lipoprotein levels in normal rats, particularly those on the high-fat, high-carbohydrate diet were not explored in this study, but are areas that will require further research.

FIG. 1. Effect of Cr3 on fasting blood glucose in Wistar rats fed normal and cafeteria-style diets.

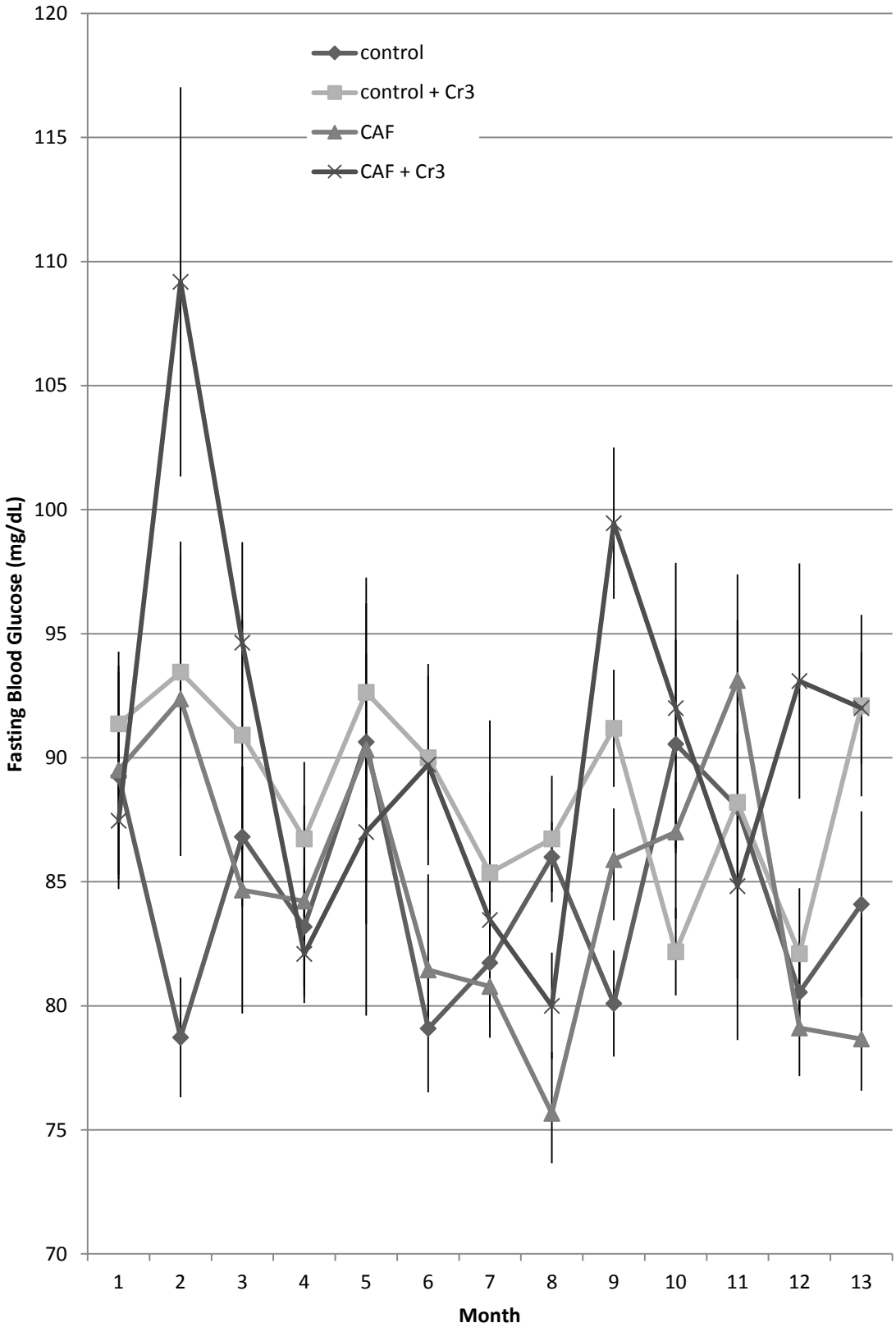
120
115
110
105
100
95
90
85
80
75
70
Fasting Blood Glucose (mg/dL)
control
control + Cr3
CAF
CAF + Cr3
1
2
3
4
5
6
7
8
9
10
11
12
13
Month

TABLE 1. Least significant difference between control and control + Cr3, and between CAF and CAF + Cr3 for area under the curve, glucose challenges

Groups Compared	Time	p Value
Control - Control + Cr3	Month 4	0.433
CAF - CAF + Cr3	Month 4	0.043
Control - Control + Cr3	Month 14	0.183
CAF - CAF + Cr3	Month 14	0.646

FIG. 2. Glucose challenge, month 4, in Wistar rats fed normal and cafeteria-style diets

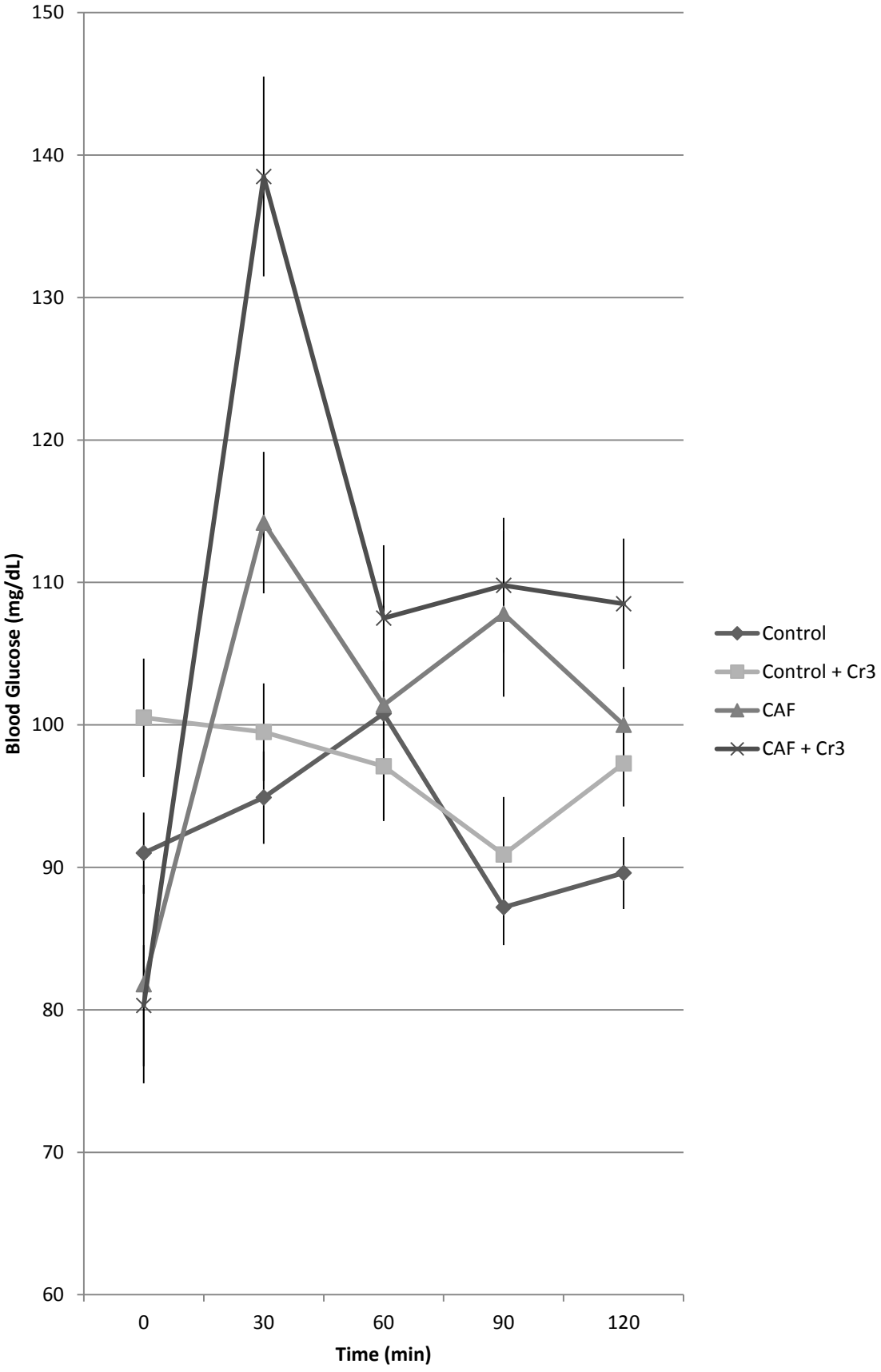
150
140
130
120
110
100
90
80
70
60
Blood Glucose (mg/dL)
0
30
60
90
120
Time (min)
Control
Control + Cr3
CAF
CAF + Cr3

FIG. 3. Glucose challenge, month 14, in Wistar rats fed normal and cafeteria-style diets

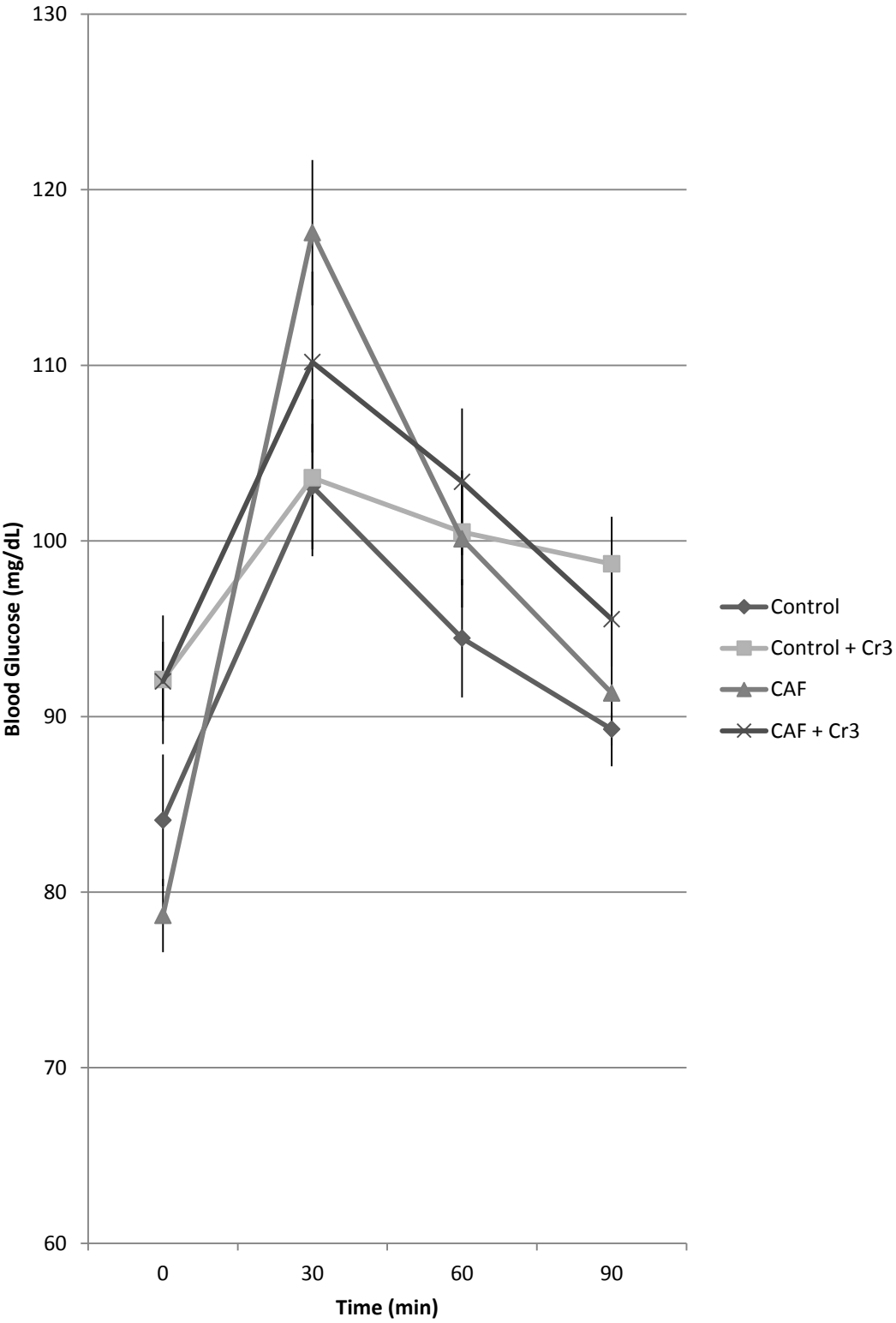
130
120
110
100
90
80
70
60
Blood Glucose (mg/dL)
0
30
60
90
Time (min)
Control
Control + Cr3
CAF
CAF + Cr3

FIG. 4. AUC for month 4 glucose challenge in Wistar rats fed normal and cafeteria-style diets

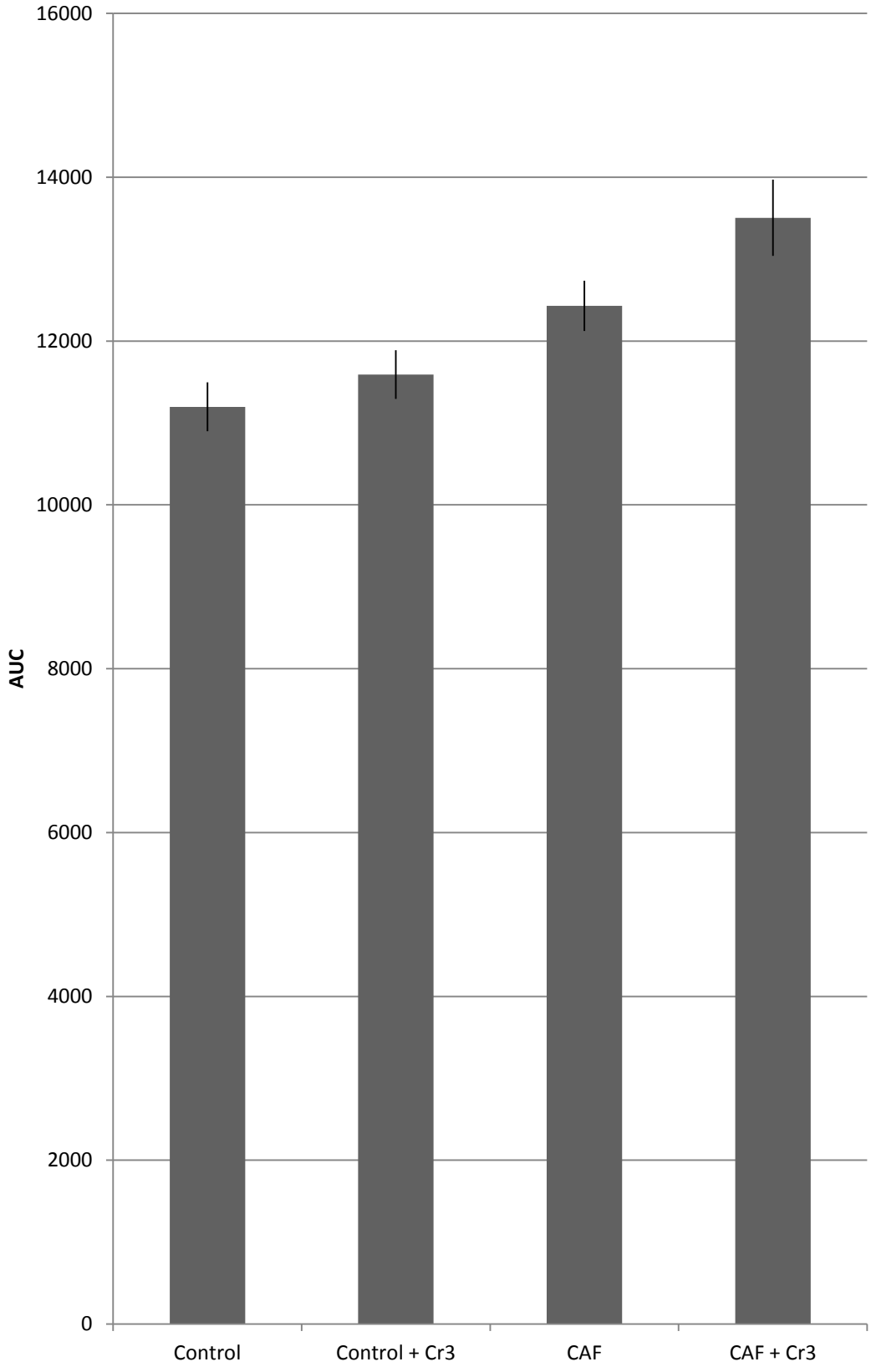
16000
14000
12000
10000
8000
6000
4000
2000
0
AUC
Control
Control + Cr3
CAF
CAF + Cr3

FIG. 5. AUC for month 14 glucose challenge in Wistar rats fed normal and cafeteria-style diets

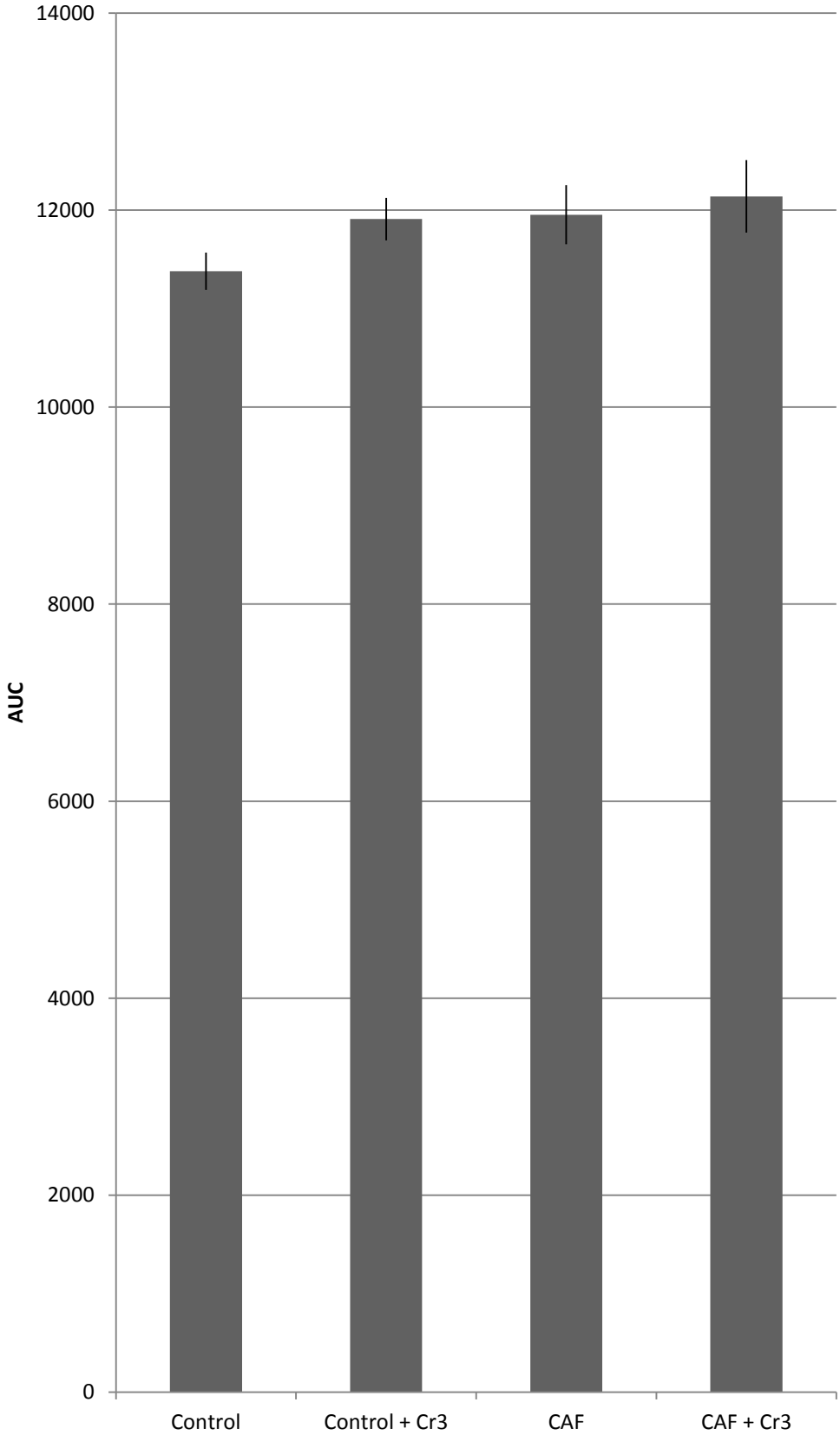
14000
12000
10000
8000
6000
4000
2000
0
AUC
Control
Control + Cr3
CAF
CAF + Cr3

FIG. 6. Insulin challenge, month 10, in Wistar rats fed normal and cafeteria-style diets

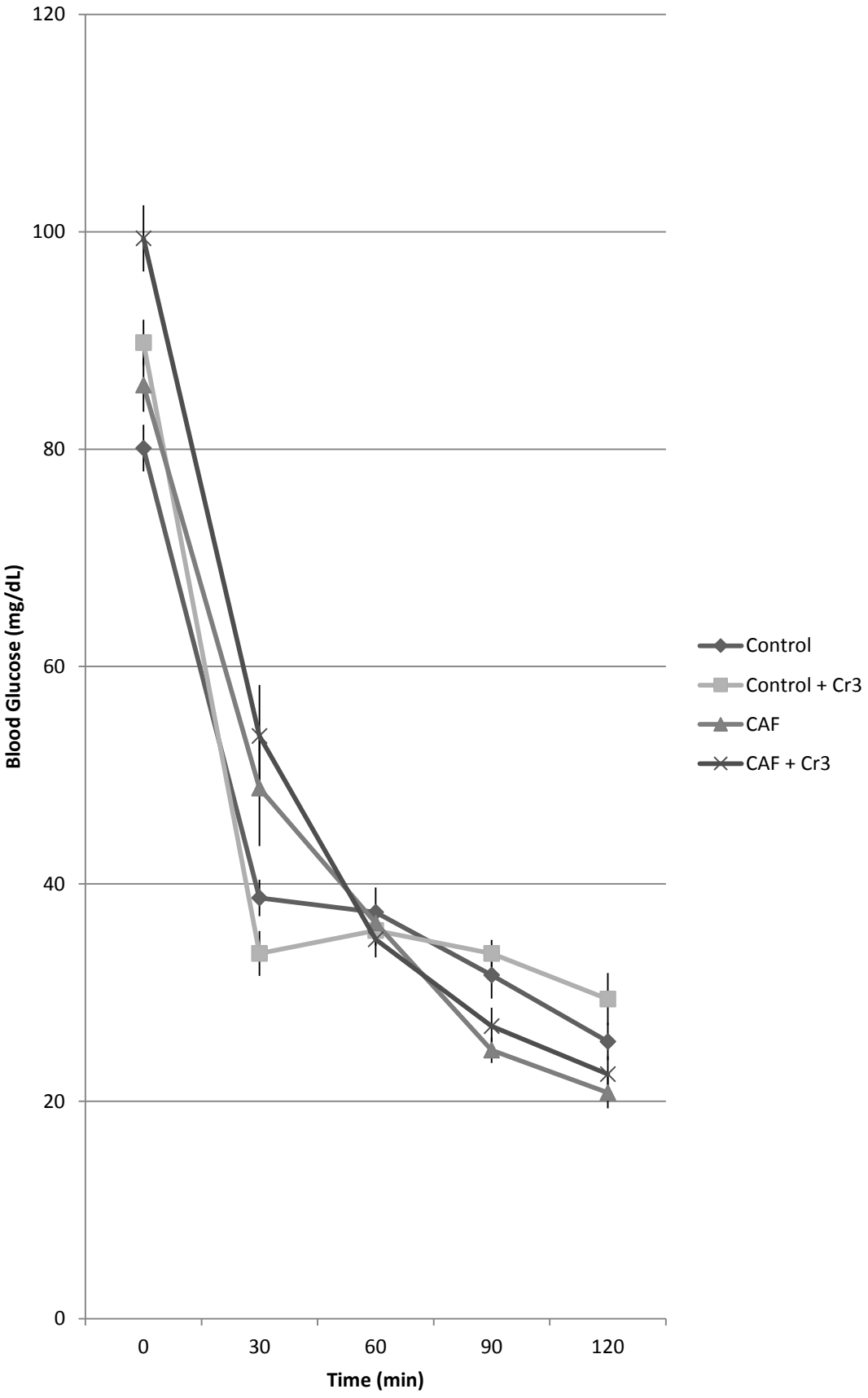
120
100
80
60
40
20
0
Blood Glucose (mg/dL)
0
30
60
90
120
Time (min)
Control
Control + Cr3
CAF
CAF + Cr3

FIG. 7. Insulin challenge, month 14, in Wistar rats fed normal and cafeteria-style diets

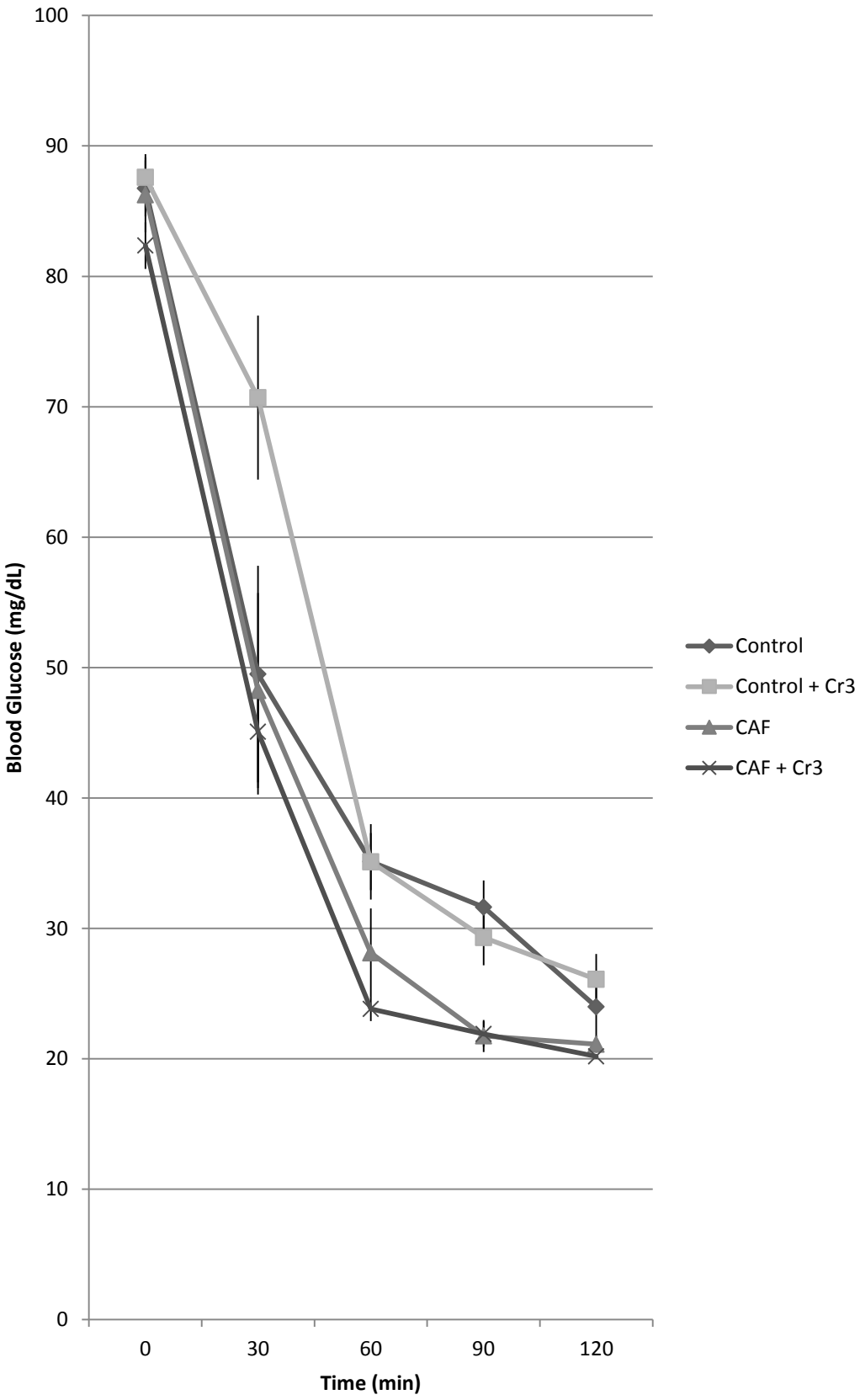
100
90
80
70
60
50
40
30
20
10
0
Blood Glucose (mg/dL)
0
30
60
90
120
Time (min)
Control
Control + Cr3
CAF
CAF + Cr3

FIG. 8. AUC for month 10 insulin challenge in Wistar rats fed normal and cafeteria-style diets

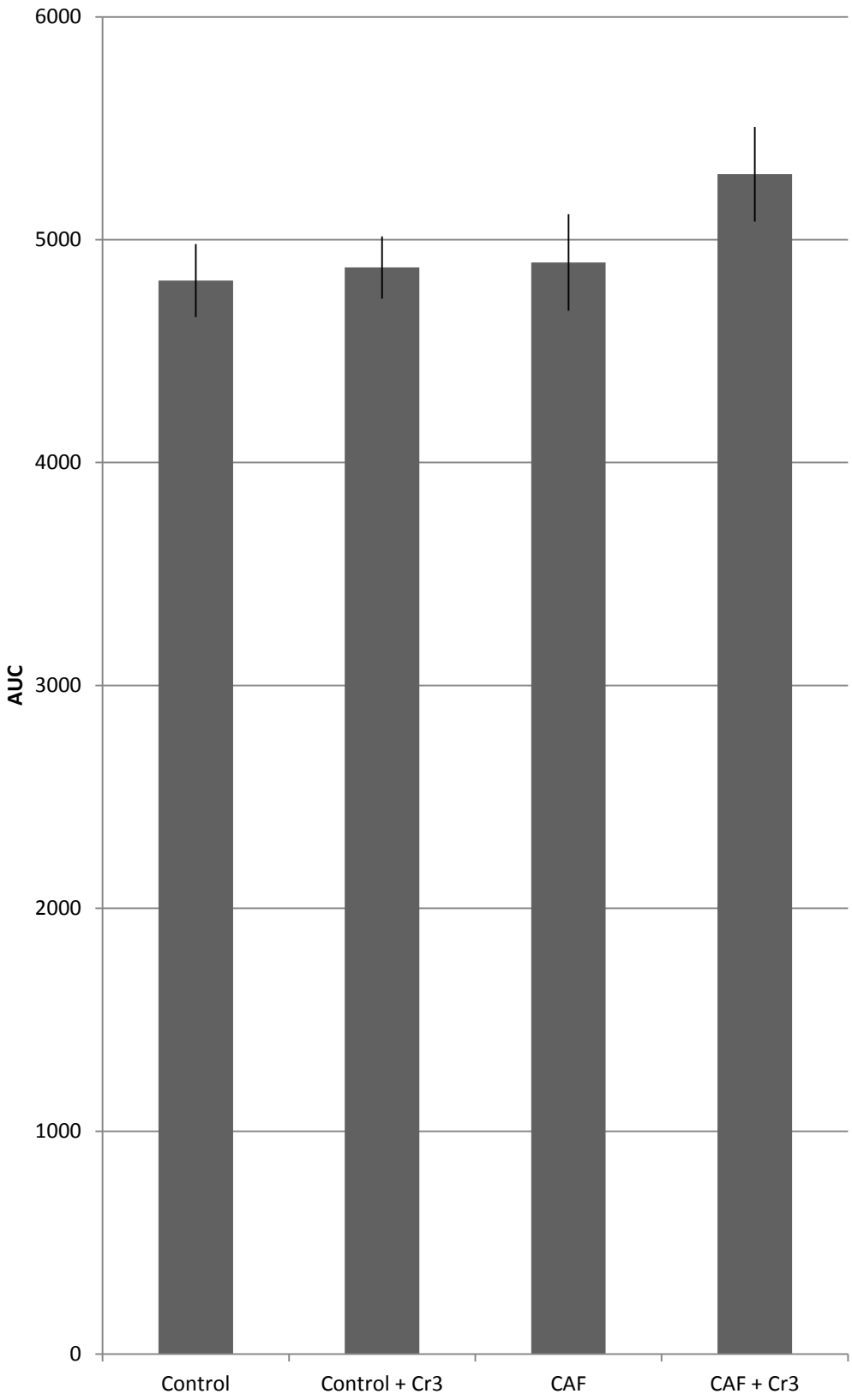
6000
5000
4000
3000
2000
1000
0
AUC
Control
Control + Cr3
CAF
CAF + Cr3

FIG. 9. AUC for month 14 insulin challenge in Wistar rats fed normal and cafeteria-style diets

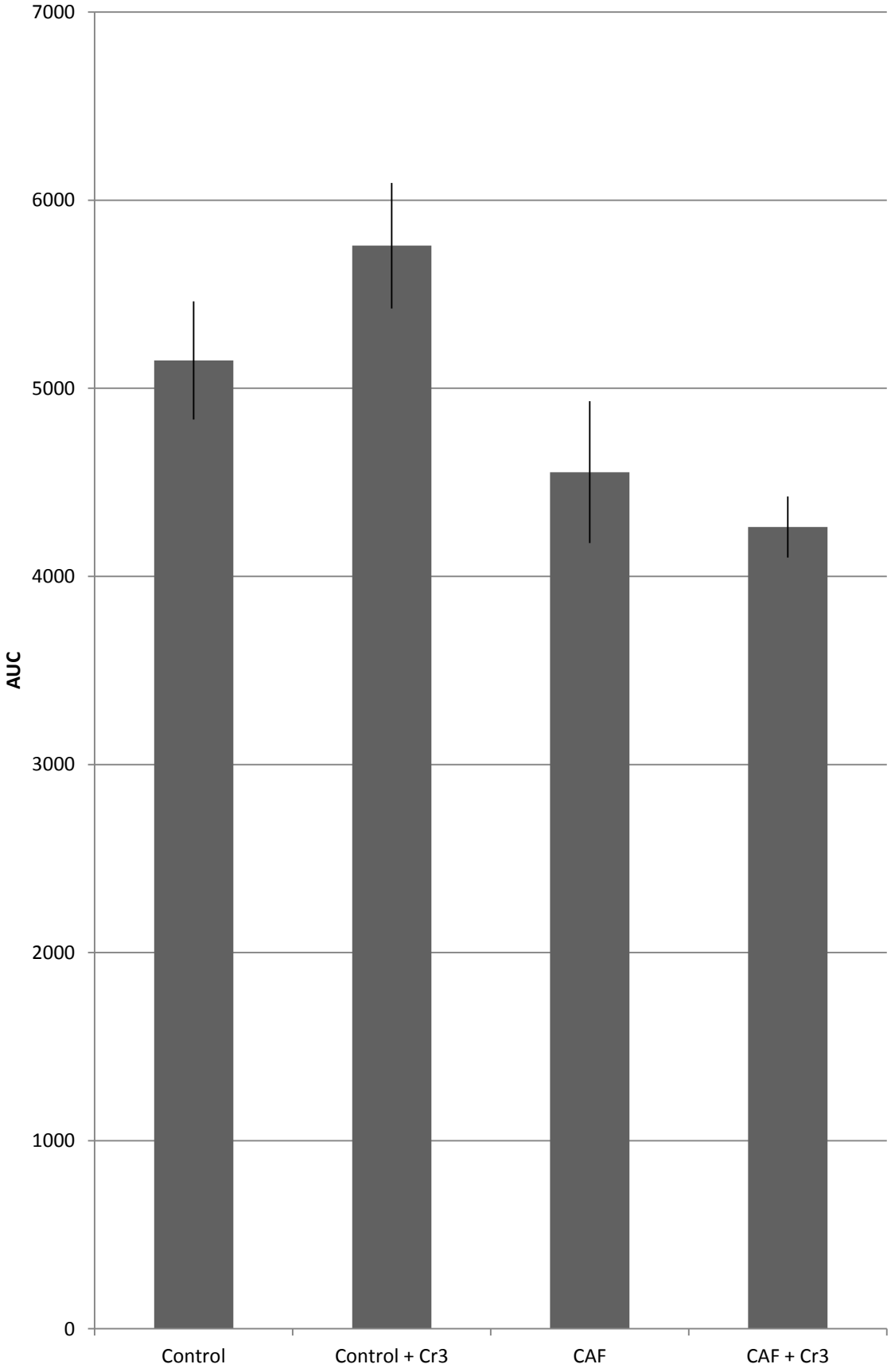
7000
6000
5000
4000
3000
2000
1000
0
AUC
Control
Control + Cr3
CAF
CAF + Cr3

FIG. 10. Effect of Cr3 on body mass in Wistar rats fed normal and cafeteria-style diets.

[a]differs significantly from all other groups ($p \leq 0.05$)

[b]differs significantly from both control groups ($p \leq 0.05$)

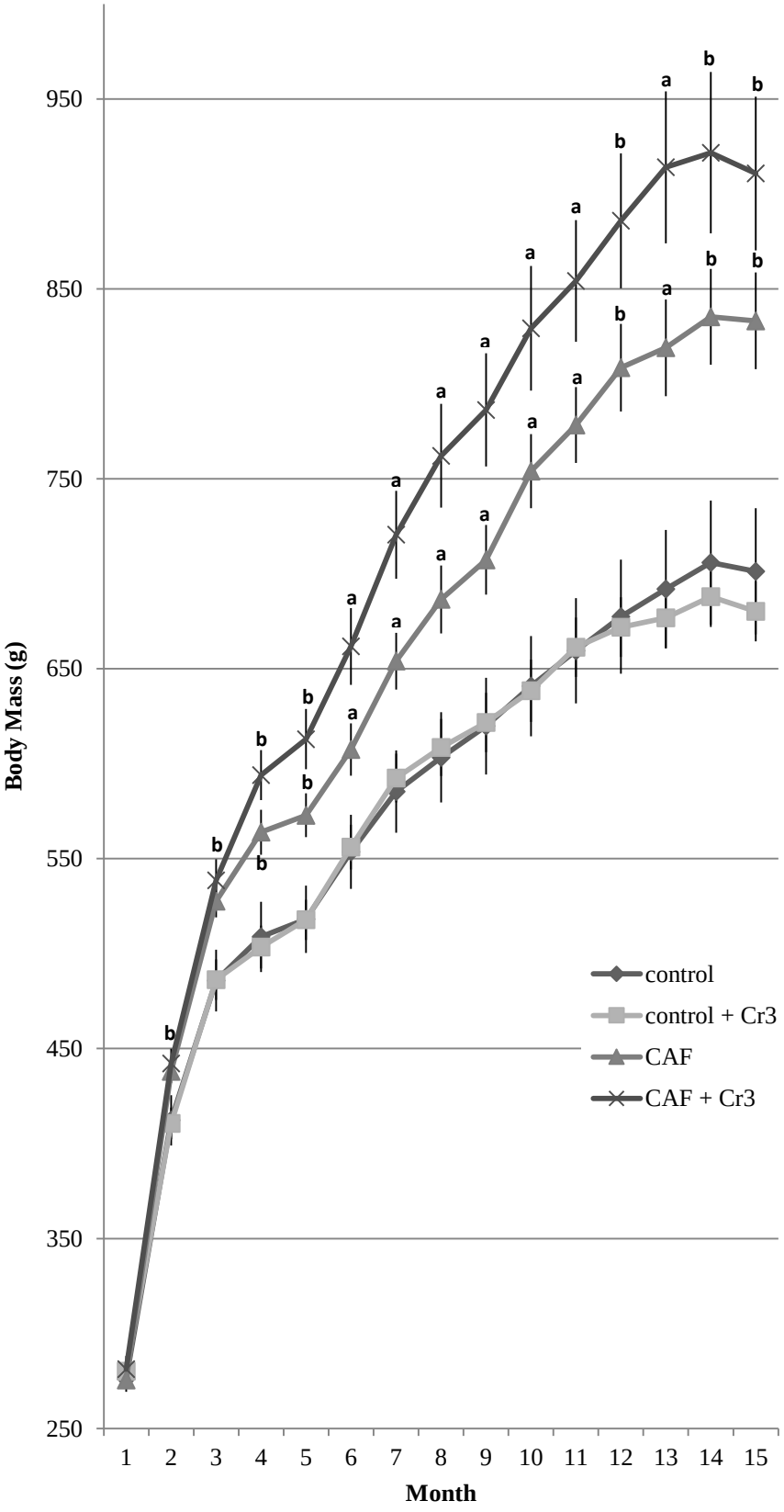
Body Mass (g)
950
850
750
650
550
450
350
250
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Month
control
control + Cr3
CAF
CAF + Cr3

TABLE 2. Effect of Cr3 on visceral measurements in Wistar rats fed normal and cafeteria-style diets.

	Control (±SEM)	Control + Cr3 (±SEM)	CAF (±SEM)	CAF + Cr3 (±SEM)
Liver (g)	25.75 ± 1.37	26.29 ± 1.08	28.05 ± 2.28	28.44 ± 1.15
L. lung (g)	2.2 ± 0.17	2.36 ± 0.12	2.27 ± 0.16	2.56 ± 0.20
R. lung (g)	2.65 ± 0.19	2.25 ± 0.15	2.00 ± 0.16	2.64 ± 0.25
Heart (g)	2.75 ± 0.18	2.60 ± 0.13	2.77 ± 0.21	2.86 ± 0.17
L. kidney (g)	2.58 ± 0.10	2.57 ± 0.12	2.78 ± 0.39	2.44 ± 0.13
R. kidney (g)	2.61 ± 0.10	2.64 ± 0.15	3.08 ± 0.41	2.44 ± 0.13
Visceral fat (g)	31.45 ± 3.26	32.33 ± 3.01	47.92[a] ± 2.83	57.18[a] ± 5.18
Body wall thickness (mm)	5.83 ± 0.30	5.85 ± 0.28	6.77 ± 0.25	6.89 ± 0.46

[a]Differs significantly from Control and Control + Cr3 ($p < 0.01$)

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