

Effects of Membrane Metallo-Endopeptidase Inhibitor Alone or in Combination with Azilsartan on Renin-Angiotensin-Aldosterone-System in Rats with Heart Failure

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ABSTRACT

Purpose: Renin-angiotensin-aldosterone-system activation associated with heart failure is one of the main determinants of health condition deterioration of the patients with heart failure. The aim of this study is to compare the effectiveness of the metallo-endopeptidase inhibitor Sacubitril alone and in combination with azilsartan in ameliorating syndromes of heart failure in rats.

Materials and Methods: twenty four rats were divided into 4 groups, each of 6 rats. The first group served as the normal control group. Heart failure was induced in the rats of the other three groups with isoproterenol 5 mg/kg/day for one week, 2nd group rats served as appositve control group, rats in the 3rd, and 4th groups were administered daily oral doses of sacubitril, and sacubitril-azilsartan combination respectively for two weeks.

Results: Heart failure induction in rats with isoproterenol showed a statistically significant increase in plasma concentration of NT-proBNP, MMP9, Renin, Troponin I, and CK-MB. A significant decrease in mean blood pressure, urine flow, glomerular filtration rate was observed.

Administration of sacubitril-azilsartan combinations showed a significant fall of MMP9, NT-proBNP, serum urea, Troponin I and creatinine. Rats that have received Sacubitril alone did not show significant changes in the mentioned parameters except for NT-proBNP and serum creatinine.

Combining sacubitril with azilsartan showed a significant increase in urine flow, glomerular filtration rate, and renin plasma level, whereas Sacubitril alone failed to show a significant change in the mentioned parameters.

Conclusion: Combination of sacubitril with azilsartan showed better efficacy to sacubitril monotherapy in improving plasma levels of cardiac biomarkers: NT-proBNP, MMP9, and troponin I levels. It has ameliorated compromised renal function through increasing glomerular filtration rate, urine flow, creatinine clearance, and urea clearance. Results revealed that the combination (azilsartan-sacubitril) showed better ameliorating impacts on syndromes of heart failure induced by isoproterenol in rats.

Keywords: endopeptidase inhibitor, sacubitril, azilsartan, heart failure, isoproterenol, renin .

التأثيرات لمثبطات متالونيندوپيپتايديز بمفردها وبالاقتران مع ازيلسارتان على رينين أنجيوتنسين ألدوستيرون في الجرذان التي تم استحداث قصور القلب لديها بواسطة التجربة

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الخلاصة

خلفية: إن تنشيط آليات تعويضية عصبية-هورمونية هو ظاهرة فسيولوجية شائعة في قصور القلب من أجل تعويض فشل القلب، والذي عادة ما يكون له تأثير متدهور على الحالة الصحية العامة. تم مؤخراً تجربة العديد من الأدوية، مثل مثبطات النيبيريلايسين ومثبطات الأنجيوتنسين، لعلاج التغيرات العصبية-الهرمونية. أجريت هذه الدراسة لتقييم فعالية الساكوبياتريل بمفرده أو بالتركيبة مع أزيلسارتان، في علاج التغيرات العصبية-هورمونية في الفئران المصابة بفشل القلب الناجم عن التجربة.

الطريقة: تم توزيع ستة وثلاثين جرذاً من الفئران الغربية (ويستر - ألبينو) بشكل عشوائي إلى ست مجموعات، بحيث يحتوى كل مجموعة على ستة جرذ. كانت المجموعة الأولى المجموعة الضابطة (لم يتم استخدام أية مواد عليها). تم استخدام حقن إيزوبرينالين داخل الصفاق من 5 مغ/كغ/يوم لمدة أسبوع واحد للحث على استحداث نماذج من قصور القلب التجريبية في الجرذ من بقية المجموعات. خدمت المجموعة الثانية كعنصر تحكم إيجابى. تلقت الجرذ فى المجموعات الثالثة والرابعة والخامسة والسادسة جرعة يومية عن طريق الفم من الساكويباتريل 30 مغ/كغ/يوم ، و الساكويباتريل- أزيلسارتان 30 و 3 مغ/كغ/يوم مع 3 مغ/كغ/يوم، و ، على التوالي لمدة أسبوعين.

النتائج: أدى تحفيز قصور القلب فى الجرذ إلى زيادة كبيرة فى دوران CK-MB و troponin-I و MMP9 و NT-proBNP و رينين واليوريا والكرياتينين فى الدم.

تم ملاحظة إنخفاضات كبيرة فى معدل الترشيح الكبيبي، وتدفق البول، وإفراز النوبان الكلى، و معدل ضغط الدم فى الجرذ التى لديها قصور القلب. أظهرت الجرذ التى عولجت بالساكويباتريل مقترنة بالأليسكيرين ، أزيلسارتان أو رامبيريل انخفاضاً إحصائياً كبيراً فى NT-proBNP ، MMP9 ، و مصل تروبونين اليوريا، و مصل الكرياتينين. إستخدام الساكويباتريل- الأليسكيرين، الساكويباتريل- أزيلسارتان أدت إلى زيادة ملحوظة فى مستوى بلازما رينين وإفراز النوبان الكلى ، وتدفق البول ، ومعدل الترشيح الكبيبي.

استنتاج: قام الساكويباتريل المقترن مع الأليسكيرين، أزيلسارتان أو مع رامبيريل بخفض العلامات الحيوية لبالزما القلب مثل CK-MB و MMP9 و NT-proBNP بشكل فعال فى الجرذ المصابة بفشل القلب. أظهرت جميع التركيبات علاجاً كبيراً لوظيفة الكلى من خلال زيادة GFR ، تدفق البول، وإفراز النوبان الكلى، وكذلك تقليل مستوى البلازما للرينين. كشفت النتائج النهائية أن هذه مجموعات التراكيب لها تأثيرات علاجية مماثلة على التغيرات العصبية-الهرمونية، ولكن لها تأثيرات أفضل من استخدام مثبطات النيبيريلاسين وحدها.

الكلمات المفتاحية: نيندوبيبتيديز ، ساكويباتريل ، ازيلسارتان ، عجز قلب .

INTRODUCTION

Heart failure is a progressive complex disorder that is described by incapability of heart to eject adequate blood to satisfy the physiological needs of our body. It is caused by defects in the heart muscles, which is leading to insufficient blood pumping to different parts of body, and this will lead to appearance of certain clinical signs such as dyspnea, edema, and fatigue.^{1,2}

Pathophysiology of heart failure includes changes in the physiology of heart. All of these changes are ending up with vasoconstriction, and increased blood volume in order to increase ventricular filling.

Heart failure is accompanied with neurohumoral changes and alterations in sympathetic supply, although under normal physiological condition these changes provide a good compensation for the load. Yet, in chronic heart failure neurohumoral changes has an important role in the deterioration of the disease. Stimulation of the renin-angiotensin-aldosterone system (RAAS) increases level of renin, plasma angiotensin I, angiotensin II, and aldosterone. Angiotensin II is known as a vasoconstrictor agent of renal afferent arterioles, which increases plasma level of circulating catecholamines, and stimulates secretion of aldosterone. Intern this increases catecholamines level stimulates sodium retention in the proximal tubules and enhances excretion of potassium.

Consequently, this will lead to increased Na and water reabsorption, and increased K excretion.³

The neurohumoral changes induce the release of natriuretic peptides (NPs) through increased stretch of the cardiac wall. NPs are hormones that regulate sodium and water excretion though promoting natriuresis and vasodilation. Main types of NPs are atrial natriuretic peptides (ANP) and brain natriuretic peptides (BNP), which are released up on increased wall stress in the atrium and ventricle respectively, in the study Pro-BNP, which is metabolite of BNP, is because it shows relative stability compared to BNP and ANP and its level is not affected by different treatments.⁴

Natriuretic peptides increase Na⁺ and water excretion; relax vascular smooth muscle, and inhibit the release and/or actions of several hormones and mediators. NPs exert their effects though stimulating NP receptors which exist in at least two subtypes, designated A and B.⁵ These peptides have relatively short circulatory duration (minutes) because they are quickly destructed by an enzyme called membrane metallo-endopeptidase (MME), which is mainly synthesized in kidneys.³

Membrane metallo-endopeptidase terminates the action of many peptides and autacoids, like NPs, angiotensin II, bradykinin, substance P, and adrenomedullin.¹

Pharmacotherapy for patients with heart failure mainly includes some of adrenergic β -receptor

blockers, angiotensin-converting enzyme inhibitors, and angiotensin receptor blockers.⁶

With the intention of improving syndromes of heart failure, few membrane metallo-endopeptidase inhibitors like sacubitril and candoxatril were designed and tested to check outcome of these medicines. Clinical trials available in this aspect are limited, although none of them has been tested to check the exact neurohumoral changes experimentally.⁷

Using membrane metallo-endopeptidase inhibitor (sacubitril) will increase plasma level of angiotensin II, that's why sacubitril is combined with ACE inhibitors or renin inhibitors to exhibit better relief of the neurohumoral changes. The aim of this study is to assess the neurohumoral effects of membrane metallo-endopeptidase inhibitor sacubitril alone and with its combination with azilsartan in treating experimentally induced heart failure in rats.

MATERIAL AND METHODS

Animals

24 female albino rats were used in this study weighing 200-280 g. The rats were obtained from the animal facility for laboratory animals in Kurdistan region of Iraq. Standard rat cages were used during the study in the animal facility of Hawler Medical University. Rats were kept with having access to water and food pallet. The facility hall was programmed on half-day light/half-day dark rounds.

Materials

Isoproterenol HCl (creative enzymes), sacubitril Calcium (MedKoo Biosciences), azilsartan medoxomil (Takeda).

ELISA rat kits for each of renin, NT-proBNP, MMP9, CK-MB, and troponin-I were obtained from Elabsciences.

Study Design

30 Wister albino rats were randomly divided into 4 groups, each group included 6 rats. Rats in group I were used as control, they have received intraperitoneal isotonic saline for one week, after that they were administered starch for two weeks. Group II holds the positive control rats in which heart failure was induced experimentally, rats in this group were injected with 5mg/kg Isoproterenol intraperitoneally for seven days for this purpose, then the rats were administered placebo for two weeks.⁸

After inducing heart failure experimentally with isoproterenol rats of the group III, and group IV, the rats of the third group were administered Sacubitril 30 mg/kg/day,⁹ whereas rats in the fourth

group were administered Sacubitril 30 mg/kg/day with Azilsartan 3 mg/kg/day,¹⁰

Blood pressure, heart rate, 24-hour urine volume, and body weight were monitored on the 1st day of the experiment in the first group. Mentioned parameters were logged on the 8th day of the experiment in the 2nd group of rats, and on the 21st day (3 hours following last dose) for III, and IV groups.

The process of urine collection was achieved with the help of specially designed urine collection cages, measuring 15 cm height and 6 cm in diameter with a funnel that was covered by a mesh, to which a collecting canister was fixed for urine collection.

All the animals were injected with Xylazine 21 mg/kg and Ketamine 45 mg/kg on the day twenty first day of the experiment,¹¹ Blood samples were collected through cardiac puncture technique. A part of collected serum was taken for biochemical assays by using ELISA. The other part of collected serum was taken to be tested for other parameters that need Cobas instrumentation.

The 24 hour collected urine samples for all rats were used to measure the glomerular filtration rate (GFR) and urine flow.

Biochemical Assays

Biochemical assays were carried out to measure: NT-proBNP, MMP9, renin, CK-MB, and troponin I in the serum with ELISA rat kit for each parameter. Urine, serum creatinine, and blood urea nitrogen (BUN) have been estimated though using s Cobas-Roche analyzer. Urine sodium and potassium were measured by using flame photometer Jenway PFP7.

Ethics Committee Approval

Approval number 180502171 is awarded to this work by the committee of the ethics in the Hawler Medical University/Pharmacy school.

Statistical Analysis

The data were represented as the mean \pm standard error (M \pm SEM). Results were calculated though using Statistical package for social sciences software and one-way analysis of variables was used to analyze the differences in all parameters. Tukey test was used to compare between groups. Whenever P value is <0.05 then difference is assumed to be significant.

RESULTS

Effects of Sacubitril Alone, and in Combination with Azilsartan on Biochemical Assays

Data showed in table 1 illustrates that the administration of azilsartan together with sacubitril for two weeks significantly lowered the raised level

of troponin I, NT-proBNP and MMP9 in rats heart failure, however, they both have increased renin plasma level.

Results show that plasma level of Troponin I is significantly reduced by combinations of azilsartan with Sacubitril with sacubitril from 0.62 ± 0.12 ng/ml to 1.65 ± 0.22 ng/ml, when they are compared to the positive control group. However, comparing it with control group, reducing troponin I level is not statistically significant, sacubitril alone also reduced troponin I level to 2.32 ± 0.28 ng/ml, and the change when it is compared with Isoproterenol pretreated rats is not significant statistically.

Results reveal that the experimentally induced failing heart group has significantly higher NT-proBNP plasma levels (980 ± 116.71 pg/ml) when it is compared to control group (658 ± 58.68 pg/ml). We have also observed that treatment group (III, and IV) efficiently reduced the level of NT-proBNP to 658 ± 58.68 pg/ml, and 376 ± 22.38 pg/ml respectively, and the reduction was significant when it was compared to the positive control.

Inducing heart failure with Isoproterenol has significantly increased the level of CK-MB to 31.55 ± 1.69 ng/ml when it is compared to the control group plasma level (13.78 ± 1.67 ng/ml). Sacubitril failed to reduce CK-MB level significantly, CK-MB levels in azilsartan groups resulted in an improvement compared to the isoproterenol pretreated group.

Inducing heart failure with Isoproterenol high dose resulted in a significant increase in the plasma level of MMP9 from 15.85 ± 0.57 ng/ml to 9.91 ± 0.43 ng/ml compared to the control group. We observed that combinations of azilsartan-sacubitril has significantly reduced MMP9 level when they were compared to the positive control group. Meanwhile the difference between both mentioned group is not significant with the control group and the level was returned back to normal.

Renin concentration was increased significantly in groups II, III, IV and V when it was compared with the control group. However, rats that had received combinations of Azilsartan-Sacubitril combination showed higher plasma renin value (748 ± 43.073 pg/ml) when they were compared to Sacubitril pretreated rats (693 ± 42.35 pg/ml).

Effects of Sacubitril Alone, and in Combination with Azilsartan on Renal Function

Results in table 2 revealed that inducing heart failure had significantly reduced GFR from 140.5 ± 14.7 ml/m/kg to 7.031 ± 1.6 ml/m/kg. Rats in different groups illustrated a statistically significant rise in GFR compared to isoproterenol-pretreated group. Blood urea nitrogen and serum creatinine were reduced in both treatment groups when it was compared to the positive control group, however

Azilsartan-Sacubitril combinations showed results that were significantly different with group II. On the other hand, sacubitril alone failed to reduce the levels of serum creatinine and blood urea nitrogen significantly compared to the positive control group. Results showed that all treatment regiments were able to increase the significantly reduced urine flow in group II, however no statistically significant difference was found between groups III and IV.

Effects of Sacubitril Alone, and in Combination with Azilsartan on Heart Rate, and Systolic and Diastolic Blood Pressure:

Mean and systolic pressure in all groups were significantly reduced when it was compared to the rats within the control group. Among all treatment groups. Unlike systolic blood pressure, a significant reduction of diastolic blood pressure was observed in all rats when its compared to the control rats.

Rats with induced HF showed higher heart rate than rats in control group. Heart rate was dropped with treatments in all the treated rat groups; however, no significant difference was observed in heart rate in rats treated with sacubitril combined with azilsartan when it was compared to control group rats (table 3).

DISCUSSION

Results of this experiment show that daily intraperitoneal injection of 5mg/kg isoproterenol for seven days has induced pathophysiological changes similar to the syndrome of heart failure in the rats. Activation of the RAAS appears to have an important role for the progression of pathophysiological changes in circulatory system and heart remodeling. This model has been used by many researchers to examine therapeutic interventions in heart failure.^{7,9}

Many mechanisms have been suggested to explain reasons behind isoproterenol-induced myocardial pathogenesis, like imbalance in oxygen demand and supply from myocytes because of hyperfunction due to the exacerbation in heart rate and cardiac muscle contraction.¹² A recent study showed that nitric oxide play an important role in heart failure. It shows that compensatory expressed amount of nitric oxide cannot maintain contractile function of myocytes, it may lead to a decreased heart rate, as well as acceleration of beta-adrenergic stimulation of myocardial force of contraction.¹³

Troponins are specific markers of cardiac cell damage. Although, the normal physiological value of cardiac troponin I that results from normal protein breakdown is very low, Troponin I plasma level is significantly higher after inducing heart failure which is practically shown in case of acute

myocardial infarction and heart failure. This rise in Troponin is probably due to the irreversible damage of cardiac myocytes. This finding is in agreement with results of Hasic coworkers,¹⁴ and results of this study showed that high dose of isoproterenol caused an increase of Troponin I levels in rats with failing hearts with a mean of 3.09 ± 0.147 ng/ml.

The inactive ProBNP after secretion is cleaved into equivalent amounts of NT-proBNP and the active BNP. Both of them are proteins released into the circulation during myocardial damage and used as important tools for diagnosis and evaluation of cardiac physiological status.¹⁵ In this research work The cardiac biomarker NT-proBNP was used and not BNP because BNP has a shorter half-life which makes NT-proBNP more practical to handle serum before making measurements, also previous works revealed that NT-proBNP serum level is not changed by membrane metallo-endopeptidase inhibitor therapy, in contrast to BNP, as its serum value shows significant changes in patients receiving membrane metallo-endopeptidase inhibitor therapy.¹⁶

Blood levels of Creatinine Kinase (CK) enzyme rises when skeletal muscles or heart cells are injured. CK-MB is the most specific isoform correlated to heart muscle injury. CK-MB is a laboratory tool for evaluating the degree of cardiac muscle injury.¹⁷ The reduced serum level of CK-MB in rats of groups III, and IV indicates decrease of the progression of cardiac cell injury. Sacubitril administration with Azilsartan seems to be the most operative in attenuating progression of cardiac muscle injuring, and it has effectively reduced NT-proBNP as well. This could be a reason behind results obtained by Sakamoto coworkers in which they showed that Azilsartan has better effect on ventricular function than Candesartan.¹⁸

Matrix metalloproteinases are group of enzymes that break matrix polypeptides and in this way it changes the outcome of many physiological and pathological conditions including myocardial infarction and congestive heart failure. The process of cardiac muscle remodeling is shown to be related to the elevated serum level matrix metalloproteinase subtype 9 that's why it's used as a biomarker for evaluating heart muscle remodeling.¹⁹ Results are indicating that both combinations are able to attenuate the process of remodeling. A study has shown that raised serum level of MMP9 is related with enlarged left ventricle and thicker ventricular wall.²⁰ also, raised serum level of MMP9 is connected to increased left ventricular hypertrophy and dysfunction following infarction.²¹

The increased serum level of the renin in heart failure resulted from activation of neurohumoral cascade, and usually has negative end impacts on the normal hemodynamic functions.²² administration of azilsartab lowers circulating renin activity although renin serum concentration increases and that is because azilsartan blocks angiotensin receptor. Eventually, this will end up with an increase in circulating renin concentration^{23,24}. These results disagree with what is stated by Cromer and Peker who stated that plasma concentration of renin decreases with renin blockers therapy.²⁵

All the mentioned parameters above indicate the reduction of work load on the cardiac muscle in Groups III, and IV rats. This can be seen in the form of lowered NT-proBNP and troponin, which are released up on incased ventricular work stress and myocardial injury. Moreover, all medications used in this study significantly reduced MMP9, however combination of Sacubitril with Azilsartan showed the ability of these agents to slow down the process of cardiac remodeling. Slowing down the process of cardiac remodeling is considered important clinically and prolongs the lifespan in patients with heart failure.²⁶

Urine flow and GFR are both significantly reduced with heart failure induction with Isoproterenol. This reduction can be attributed to the fact that physiological compensation in heart failure is not enough to make up for the defective cardiac function.²⁷ Add to that, urea and creatinine can be efficiently excreted by kidneys and this will lead to an increase in serum levels of both creatinine and blood urea nitrogen and creatinine, and this indicates a compromise in the renal function.²⁸

Membrane metallo-endopeptidase inhibitors like Sacubitril stop breakdown of NPs, and since NPs stimulates sodium excretion, enhances creatinine and urea clearance and, increases GFR, and urine flow. These will reduce the elevated serum levels of urea and creatinine. Adding a renin inhibitor or angiotensin receptor blocker will further ameliorates compromised renal function through inhibiting production or the vasoconstricting effect of Angiotensin II and the possible reduction in aldosterone secretion¹¹.

Due to the failure of heart to pump blood effectively due to the diminished heart function that is induced by Isoproterenol, all the treatment groups (II, and IV) stimulate natriuresis and this will lead to additional drop down in the blood pressure. A reduction in heart rate was observed in all treatment groups. These changes urge that inhibiting RAAS system together with membrane metallo-endopeptidase inhibition gives rise to better physiological changes in heart failure than using sacubitril alone.

Previously, the desirable effects of combination of valsartan with sacubitril was described and approved to be used in heart failure, further study is needed to compare effects of sacubitril/ valsartan combination and sacubitril/ azilsartan combination to point fine differences between both combinations in order to implement results in clinical practice.²⁹

CONCLUSION

Combination of Sacubitril with azilsartan combination in improving plasma levels of cardiac biomarkers: NT-proBNP, MMP9, and Troponin I levels. It has ameliorated compromised renal function in heart failure through increasing glomerular filtration rate, urine flow, creatinine clearance, and urea clearance. Results revealed that combination of azilsartan-sacubitril showed better ameliorating impacts on heart failure syndromes when it is compared to sacubitril alone therapy in rats with isoproterenol induced heart failure.

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Disclosure

The author reports no conflicts of interest in this work

Data availability of

The data sets used for this study are available from on demand.

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Table 1 Effects of sacubitril, and azilsartan-sacubitril on cardiac and renal biomarkers

Parameter	Control	Heart failure	Sacubitril	Sacubitril and Azilsartan
Troponin-I ng/ml	0.62±0.12 4 ^a	3.09±0.147 c	2.32±0.28 5 ^{bc}	1.65±0.22 8 ^b
NT-proBNP pg/ml	356±27.63 1 ^a	980±116.7 31 ^c	658±58.68 b	376±22.38 a
MMP9 ng/ml	9.91±0.43 a	15.85±0.57 b	12.45±1.1 a	10.25±0.6 4 ^a
CK-MB ng/ml	13.78±1.6 7 ^A	31.55±1.69 b	28.71±3.1 2 ^b	21.83±3.5 8 ^{ab}
Renin Pg/ml	408±11.45 6 ^a	736±45.88 5 ^b	693±42.35 b	748±43.07 3 ^b

Different letters indicate statistically significant differences; the role of chance is excluded when P < 0.05.

Values are expressed as mean±SEM

Table 2 Effects of sacubitril and azilsartan-sacubitril on renal function

Parameter	Control	Heart failure	Sacubitril	Sacubitril and Azilsartan
GFR ml/h/kg	140.58±1 4.7 ^b	7.031±1. 6 ^a	207.02±20 .31 ^b	190.42±24 .49 ^b
Blood urea nitrogen mg/dl	32.5±1.0 5 ^a	52.1±1.5 7 ^b	47.2±2.13 ^a b	35.93±7.8 ^a
Serum creatinine mg/dl	0.37±0.0 2 ^a	0.92±0.0 4 ^c	0.498±0.0 2 ^{ab}	0.66±0.04 ^b
Urine flow ml/h/kg	0.525±0. 09 ^{ab}	0.2761±0 .06 ^a	1.0607±0. 23 ^b	1.0872±0. 28 ^b

Different letters indicate statistically significant differences; the role of chance is excluded when P < 0.05.

Values are expressed as mean±SEM

Table 3 Effects of Sacubitril and Azilsartan- Sacubitril on arterial blood pressure and heart rate

Parameters	Control	Heart failure	Sacubitril	Sacubitril and Azilsartan
Systolic blood pressure mm Hg	116.16±1.6 ^a	104.16±3.45 ^{ab}	91±1.34 ^b	93.66±7.32 ^b
Diastolic blood pressure mm Hg	85.5±0.56 ^a	72.5±1.8 ^b	66.3±0.71 ^b	68.5±3.8 ^b
Mean blood pressure mm Hg	97.3±0.76 ^a	83.5±2.6 ^b	74.3±0.8 ^b	76.6±4.9 ^b
Heart rate beat/minute	355±18.2 ^a	505.8±15.15 ^c	427±8.56 ^b	365.3±12.3 ^{ab}

Different letters indicate statistically significant differences; the role of chance is excluded when $P < 0.05$.

Values are expressed as mean±SEM

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