

UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

JOÃO VITOR TADASHI COSIN SHINDO

**Ostarine avoids masseter atrophy caused by changes
in the diet consistency**

**Ostarina (SARM) evita atrofia do músculo masseter
causada por mudanças na consistência da dieta**

BAURU
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Orientador: Prof. Dr. André Luís Shinohara

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Prof. Dr. **FÁBIO OLIVEIRA MACIEL**
UFAM




Prof.^a Dr.^a **CLAUDIA CRISTINA BIGUETTI**
UT Dallas

Prof. Dr. **JESUS CARLOS ANDREO**
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Prof. Dr. **ANDRÉ LUIS SHINOHARA**
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ERRATA

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ABSTRACT

Ostarine avoids masseter atrophy caused by changes in the diet consistency

Modern eating habits, which softer and processed foods predominate in, requiring less effort to chew and have been correlated with an increase in the incidence of malocclusion and problems in the temporomandibular joint (TMJ) also, reducing the cross-sectional area (CSA). This vital muscle fiber variable indicates muscle function and strength. Selective androgen receptor modulators (SARMs) were developed to treat muscular and skeletal system problems to replace traditional androgens. This study aimed to verify if the use of ostarine associated with a soft-diet could exert the same anabolic and anti-catabolic effects on the masseter muscle described in the literature. Fifteen six-months age C57BL/WT mice were used and divided into three groups and fed on either a solid (Control group - CG) or a soft-diet (Soft Diet Control Group - SDCG) with and without ostarine (Ostarine Group - OG). As a result, the soft-diet (SDCG) showed a lower median than the hard-diet (CG), but without statistical differences. However, the OG associated with a soft-diet presented a higher CSA than the SDCG with a statistical difference. Although there was no statistical difference between CG and SDCG group, slight atrophy occurred, and the use of ostarine (OG) reversed this process, becoming equal to the CG with a hard-diet.

Keywords: Masseter Muscle; Androgens; Atrophy; Diet Consistency; Nutrition

RESUMO

Ostarina evita atrofia no músculo masseter causada por mudanças na consistência da dieta

No mundo contemporâneo, é comum uma alimentação predominante em alimentos processados e macios, que requerem menor esforço de mastigação, havendo assim, uma correlação com o aumento da má-oclusão e problemas na articulação temporomandibular (ATM), bem como uma redução da área de secção transversa das fibras musculares dos músculos mastigatórios. A área de secção transversa é um importante parâmetro para avaliarmos a função muscular, bem como a sua força. Moduladores seletivos do receptor androgênico (SARMs), foram desenvolvidos para tratar disfunções musculoesqueléticas na tentativa de substituir os andrógenos tradicionais já usados na medicina, como a testosterona. Este estudo, objetivou-se em verificar se a ostarina (SARM) associada com uma dieta macia, poderia exercer os mesmos efeitos anabólicos e anti-catabólicos no músculo masseter como descrito em outros músculos na literatura. 15 camundongos C57BL/WT com 6 meses de vida foram usados e divididos em 3 grupos de acordo com a consistência da dieta e utilização ou não do SARM. Grupo Controle – Control Group – (CG) – consistência sólida, Grupo Dieta Macia Controle – Soft Diet Control Group – (SDCG) – consistência macia sem ostarina e Grupo Ostarina – Ostarine Group – (OG) – consistência macia com adição do sarm ostarina. Como resultado, o grupo dieta macia controle (SDCG) teve uma menor média da área de secção transversa comparado ao grupo controle, contudo, sem diferença estatística. Entretanto, o grupo ostarina (OG), apresentou uma maior área de secção transversa do que o grupo dieta macia controle (SDCG) com diferença estatística. Embora não tenha tido diferença estatística entre o grupo controle e o grupo dieta macia controle, uma sutil redução da área de secção transversa aconteceu e o uso da ostarina (OG), reverteu esse processo se igualando ao grupo controle com dieta de consistência sólida.

Keywords: Masseter; Andrógenos; Atrofia; Consistência da Dieta; Nutrição

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1- ARTICLE

The article presented in the present dissertation was written according to the formatting guidelines for submission to the Journal of Morphological Sciences (JMS)

The article title is: "Ostarine avoids masseter atrophy caused by changes in the diet consistency". It was already accepted for publication in the JMS. The acceptance letter will be annexed.

1.1 ARTICLE – “Ostarine avoids masseter atrophy caused by changes in the diet consistency”.

Abstract

Introduction: Modern eating habits, which softer and processed foods predominate in, requiring less effort to chew and have been correlated with an increase in the incidence of malocclusion and problems in the temporomandibular joint (TMJ) also, reducing the cross-sectional area (CSA). This vital muscle fiber variable indicates muscle function and strength. Selective androgen receptor modulators (SARMs) were developed to treat muscular and skeletal system problems to replace traditional androgens. This study aimed to verify if the use of ostarine associated with a soft-diet could exert the same anabolic and anti-catabolic effects on the masseter muscle described in the literature.

Material and Methods: Fifteen six-months age C57BL/WT mice were used and divided into three groups and fed on either a solid (Control group - CG) or a soft-diet (Soft Diet Control Group - SDCG) with and without ostarine (Ostarine Group - OG).

Results: As a result, the soft-diet (SDCG) showed a lower median than the hard-diet (CG), but without statistical differences. However, the OG associated with a soft-diet presented a higher CSA than the SDCG with a statistical difference.

Conclusions: Although there was no statistical difference between CG and SDCG group, slight atrophy occurred, and the use of ostarine (OG) reversed this process, becoming equal to the CG with a hard-diet.

Keywords: Masseter Muscle; Androgens; Atrophy; Diet Consistency; Nutrition

Ostarine avoids masseter atrophy caused by changes in the diet consistency

João Vitor Tadashi Cosin Shindo¹, Júlia Pedrozo Bellini¹, Cláudia Cristina Biguetti²

Maira Couto¹, Karina Torres Pomini¹, Mariza Akemi Matsumoto³, Iris Santos

German¹, Jesus Carlos Andreo¹, Rogério Leone Buchaim¹, André Luís Shinohara¹.

¹ Department of Anatomy, Bauru School of Dentistry, University of São Paulo, FOB-USP, São Paulo, Brazil, ² Department of Bioengineering of the University of Texas at Dallas(UTD), TX, ³ Department of Basic Sciences, School of Dentistry, São Paulo State University (UNESP), Araçatuba, Brazil.

Introduction

The complex interaction between the genotype and the environment can alter the craniofacial morphology and generate adaptations in the jaw structure and in the physiological overload of chewing ^{(1),(2)}. The alteration of external stimuli, such as modifying the diet consistency, alters the mass of masticatory muscles ^{(3),(4)}. Modern eating habits, which softer and processed foods predominate in, requiring less effort to chew, have been correlated with an increase in the incidence of malocclusion and problems in the temporomandibular joint (TMJ) ^{(5),(6)}.

A soft diet reduces the demand on masticatory muscles and reduces the overload applied to the jaw during the mastication process, causing bone remodeling similar to that found in individuals with partial or total edentulism ^{(7),(8)}. Changing the food consistency reduces the cross-sectional area, a vital muscle fiber variable that indicates muscle function and strength ^{(9),(10)}. Urushiyama et al., demonstrated in their work that during one week under a soft consistency diet, the smaller diameter of the masseter muscle fibers is reduced by 19% compared to animals with more solid consistency ⁽¹¹⁾.

The relation between masticatory muscles and nutritional status has been correlated ⁽¹²⁾. A perfectly functioning oral cavity, associated with good nutritional intake, especially protein, is crucial to treat and prevent sarcopenia and malnutrition ^{(13),(12)}. The low protein intake in elderly individuals may result from problems of the oral cavity, such as low masticatory function, even decreasing the appetite ⁽¹⁴⁾. Malnutrition reduces the daily activities of the elderly and increases the risks of infection and mortality ⁽¹⁵⁾.

Selective androgen receptor modulators (SARMs) were developed to treat problems in the muscular and skeletal system in order to replace traditional

androgens, which generate known adverse effects on the liver, prostate, heart, and skin ⁽¹⁶⁾.

SARMs are described in the literature as substances capable of increasing muscle mass and bone density in several experimental and clinical trials ^{(17),(18),(19)}. Among all, ostarine has the most well-documented studies and effects, proving be safe for human use ⁽²⁰⁾. However, so far, no experimental trial has been performed using this class of substances, especially ostarine, to observe its effects on the muscles of mastication.

Because of the embryological differences between the masticatory muscles and the limbs ⁽²¹⁾, we aimed to verify if the use of ostarine associated with a soft-diet, could exert the same anabolic and anti-catabolic effects on the masseter muscle described in the literature. Thus, if a positive outcome is observed, this substance may be used to prevent or even reverse the loss of muscle mass in the stomatognathic system and assist in malocclusion and orthognathic treatments.

Materials and Methods

Animals

Fifteen six-months age C57BL/6 WT mice were used, according to previous studies ⁽¹¹⁾, which were purchased from the animal laboratory of the Ribeirão Preto campus of the University of São Paulo. The animals were housed in conventional cages containing five animals each at the Animal Laboratory of Bauru School of Dentistry – University of São Paulo, with feeders and drinkers "ad libitum" (irradiated feed – Nuvilab rodents and filtered water) at temperature-controlled rooms (22-25°C). All experiment procedures in the animals were conducted with the approval of the Institutional Review Board in Animal Studies of the Bauru School of Dentistry, University of São Paulo (Protocol: CEPA – 001/2018).

Experimental design:

The division of the groups was done according to the consistency of the diet in the presence or not of the ostarine: Control Group (CG) – solid consistency; Soft Diet Control Group (SDCG) – soft consistency diet (Figure 2) which was prepared at from the standard ration (Nuvilab) under a 2:5 water mixture used by ⁽²²⁾ and Ostarine Group (OG) – soft consistency diet + administration of ostarine (SARM) at a dosage of 10 mg/kg/day (Enhanced Chemicals, MK2866), according to previous studies ⁽²³⁾.

Figure 1: Illustration of the experimental group's design. The authors prepared them.

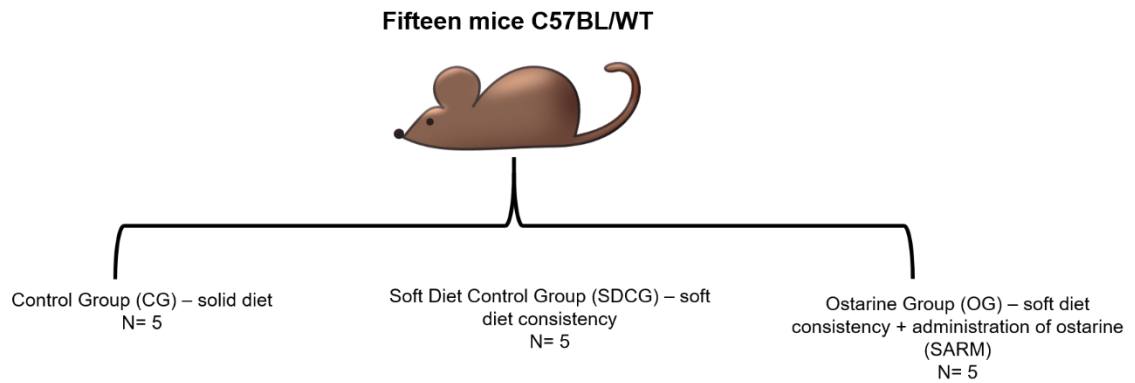


Figure 2: Soft diet prepared from Nuvilab feed under a 2:5 water mixture ⁽²²⁾.

Ostarine was administered once a day and orally for four weeks as it has excellent oral bioavailability ⁽²⁴⁾. The drug was offered using a micropipette (LabMate Pro) to avoid possible esophagus aspiration or perforation and reduce more significant stress to the mice.

At the end of the experiment, the animals were killed according to the protocols approved by the Guidelines for the Care and Use of Laboratory Animals. We dissected the superficial masseter muscle, which was immediately frozen in liquid nitrogen and stored at -80°C for later histological analysis.

Histological Processing of Frozen Muscles

After the mice euthanasia, the superficial masseter muscle was removed and embedded into the OCT (Optimal Cutting Temperature) (Tissue-Tek; Sakura Finetek, Torrance, CA, USA) and frozen in liquid nitrogen and stored at -80°C . All the samples were brought to -22°C , and serial transverse sections ($8\mu\text{m}$) were made with a cryostat (Leica 1850).

Hence, histological slides were stained with hematoxylin and eosin (H&E) to measure the muscle fiber's cross-sectional area (CSA). The microscope (model Olympus BX50) with 40x magnification was used to capture the images at the anatomy department of Bauru School of Dentistry, University of São Paulo (FOB-USP). We used the SigmaProScan 5.0 software, bypassing the perimeter of each fiber individually from each muscle, from each animal from the group to evaluate the muscle fiber's CSA. We measured 220 muscle fiber's from each sample from each animal per group.

Statistical Analysis

Given this, to evaluate the quantitative variables and verify the difference between the groups, the Kruskal-Wallis test was used, followed by the Tukey-test for multiple comparisons between them. Values of $p < 0,05$ were considered significant.

Results

The perimeter of the fibers of the masseter muscle was traced to obtain the cross-sectional area (CSA), and compared to the control group with a hard-diet (CG), the group with a soft-diet (SDCG) showed a lower median, but without statistical differences. However, the ostarine group (OG) associated with a soft-diet presented a higher CSA than the soft-diet group control (SDCG) with a statistical difference. Indeed, although there was no statistical difference between the CG and SDCG group, slight atrophy occurred, and the use of ostarine (OG) reversed this process, becoming equal to the control group (CG) with a hard-diet (Figure 3).

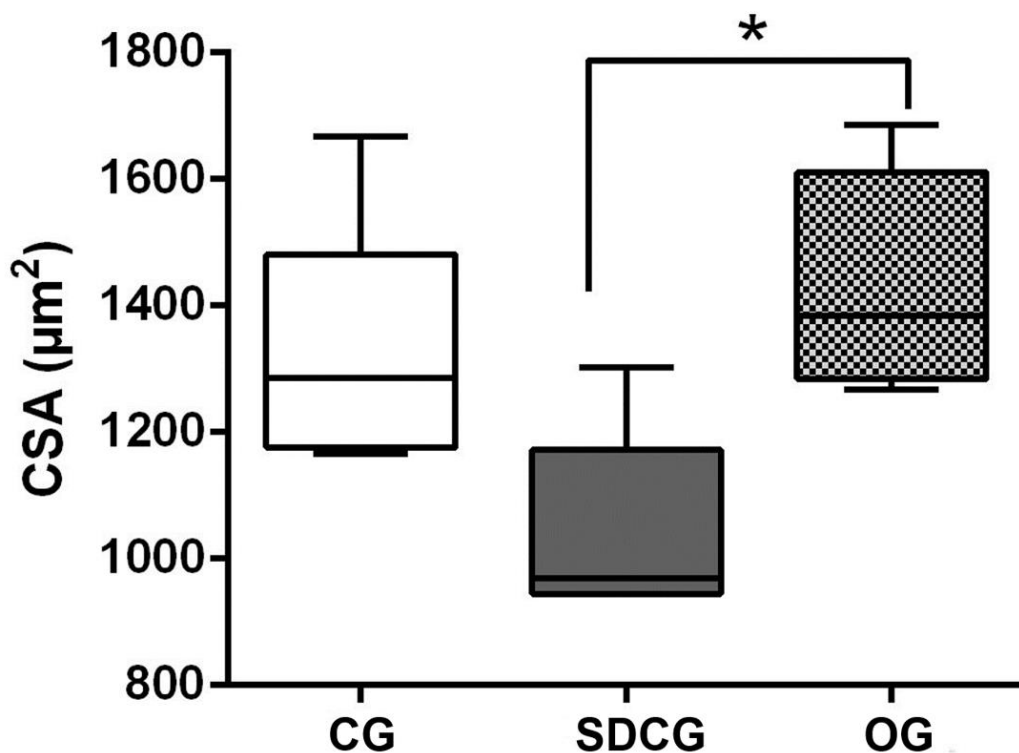


Figure 3: Mean cross-sectional area (μm^2) of the superficial masseter. Values are means + S.D. Significant differences $*P < 0,05$.

Discussion

The cross-sectional area (CSA) of the muscle fiber is an important variable that indicates muscle function and strength ⁽⁹⁾. It is reported that the changes in the consistency of the food reduce this variable ⁽¹⁰⁾. Thus, in our study, we sought to evaluate the effect of changing the consistency of the diet and its impact on the stomatognathic apparatus with and without the use of ostarine, a selective androgen receptor modulator (SARMs).

In this context, reports show that the masseter muscle is one of the main muscles activated during chewing and the bite ⁽²⁵⁾. Thus, the consistency of a soft-diet, typical in the modern days, would reduce the overload on the masticatory muscles, having a direct relationship with craniofacial changes and higher rates of malocclusion ⁽²⁶⁾. In our study, despite not being statistically significant, the CSA of the SDCG was lower than the CG (Figure 3), a result similar to that from another study ⁽²⁷⁾. However, the group with a soft-diet associated with ostarine (OG), prevented the mild atrophy caused by the changes in the consistency of the diet (Figure 3), exerting its anti-catabolic action as described in other studies ^{(28),(29)}.

As a result of the reduction in the overload generated by the contraction of the masseter muscle on the bite force, generates a remodeling in this muscle, which is the reduction in the CSA of its muscle fibers, which will impact the functionality of this muscle ⁽²⁵⁾. The activation of the ubiquitin-proteasome system (UPS) is responsible for 70-90% degradation of misfolded or damaged proteins, is the mechanism by which this occurs through the unloading on the masseter muscle by changing the diet consistency ^{(30),(31), (32)}.

Hence, no other work had used ostarine or any other selective androgen receptor modulator (SARMs) in masticatory muscles until now. Our study showed

that ostarine exerts anti-catabolic actions in the same way that it does on the muscles of the limbs ⁽²⁰⁾.

On the other hand, our study presented a limitation, which we believe to have been the reason, that the atrophy caused by the modification of the consistency of the diet, was not as reported by other studies ^{(11),(2),(33)}. Although they have already reported that many results involving this model of atrophy are not necessarily consistent ⁽³⁴⁾, we believe that the non-observance of significant atrophy may be related to the ratio administered to the animals. The ratio used in this work was described by ⁽³⁵⁾ and, compared to the feed used in some studies, ours offers 13% more calories in 100 g ^{(11),(2),(36)}. We believe that this has been a limiting factor in our work since the regulatory pathways of hypertrophy and atrophy, such as Akt/FoxO/UPS, are also regulated by caloric intake ⁽³⁷⁾. Therefore, future studies will be carried out using a normocaloric diet to verify and, if confirmed, remove this bias.

Conclusions:

Although our study has had presented a limitation, we believe that ostarine exerts anti-catabolic actions in the same way that it does on the muscles of the limbs. However, future studies must be carried out using a normocaloric diet to verify and, if confirmed, remove the bias that may affect the caloric intake of the animals.

Conflict of interest

None declared.

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Author contributions

Shindo, JVTC, Bellini, JP, Bigueti, CC, Couto, M, helped to designed the experiments also performed the experiments and collected data. Matsumoto, MA provided the resources. Andreo, JC, Buchaim, RL and Shinohara, AL, supervised the project. Shindo, JVTC, Pomini, KT, German, IJS, contributed to writing the paper. All authors reviewed and approved the manuscript.

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ANNEXES

ANNEXES

Annex A: Approval of Animal Ethical Committee



Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Uso de Animais

CEUA-Proc. Nº 001/2018

Bauru, 20 de fevereiro de 2019.

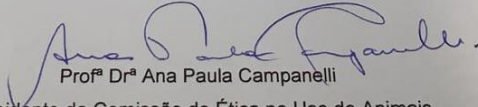
Senhor Professor,

Informamos que a proposta de pesquisa intitulada "*Influência do modulador seletivo do receptor androgênico Ostarina sobre o aparelho estomatognático em camundongos frente à administração de uma dieta pastosa*", registrada sob CEUA-Proc. Nº 001/2018, tendo Vossa Senhoria como Pesquisador Responsável, que envolve a utilização de animais (roedores), para fins de pesquisa científica, foi analisada e considerada APROVADA em reunião da Comissão de Ética no Uso de Animais (CEUA), realizada no dia 15 de fevereiro de 2019.

Vigência do projeto:	Mar/2019 a Fev/2021
Espécie/Linhagem:	Camundongos isogênico C57BL/6
Nº de animais:	45 (quarenta e cinco)
Peso/Idade	25g a 30g /6 meses
Sexo:	Macho
Origem:	Biotério Central da Prefeitura do Campus de Ribeirão Preto-USP

Esta CEUA solicita que ao final da pesquisa seja enviado um Relatório com os resultados obtidos para análise ética e emissão de parecer final, o qual poderá ser utilizado para fins de publicação científica.

Atenciosamente,



Profª Drª Ana Paula Campanelli
Presidente da Comissão de Ética no Uso de Animais

Dr. Luis Shinohara
Coordenador do Departamento de Ciências Biológicas

R. D. C. M. S. P. Bauru, SP - CEP 17012-901 - C.P. 73

Annex B: Certificate of English Review

09/20/21

Certification of English Review

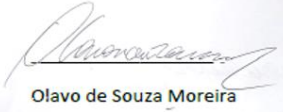
To whom it may concern,

My name is Olavo de Souza Moreira (CNPJ: 34.852.360/0001-33) and I am a Native English-speaking American residing in Brazil. I have been executing English reviews for Scientific Articles for various foreign authors in the field of Dentistry, Speech Pathology and Audiology and other Sciences since 2017.

This letter is to certify that I have performed an English review on the 20th of September, 2021 on an article entitled "**Ostarine avoids masseter atrophy caused by changes in the diet consistency**".

I am confirming that I have edited the Scientific Abstract that is being submitted with this letter of certification, and in my professional opinion, deem it noteworthy for consideration for publication.

Sincerely,



Olavo de Souza Moreira

Annex C: Acceptance Letter**Article Acceptance**

Dear Dr. João Vitor Tadashi Cosin Shindo,

It is a pleasure to inform you that your article entitled "Ostarine avoids masseter atrophy caused by changes in the diet consistency" was accepted for publication in the Journal of Morphological Sciences.

The manuscript files are now under the process of PDF production and the article will be available online no longer than January 2022.

Thank you for your valuable contribution to our journal and congratulations on your publication!

Best regards,

Valéria Paula Sassoli Fazan
Editor-in-Chief
Journal of Morphological Sciences
