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Expression of Androgen Receptors in Abating Age-Related Temporomandibular Muscles Dysfunctions in Female Albino Wistar Rats

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Abstract

Dysfunctions of Muscles of Mastication (MM) are commonly associated with facial pain, and it is a common medical condition in women's reproductive health. Hypothetically, sex hormones could be considered an underlying cause for this dysfunction, but few studies were done to explore sex hormones receptors in MM. The aim of the present study is to explore the effect of both age and sex on the expression of estrogen and androgen receptors in muscles of mastication. Eighty rats were randomly assigned into four groups. Group-12F, group-12M, group-24F and group-24M. After rats were sacrificed, MM were removed for histological and immunohistochemical examinations. Regardless age and sex, there was a weak expression of estrogen receptors (α,β) in all muscles. In group-24M, expression of androgen receptors in MM was significantly higher than that of other groups. In conclusion, the present study sheds the light on the age-related increased expression of androgen receptors in male albino wistar rats which could protect against temporomandibular muscles dysfunctions. Further studies are needed to evaluate this hypothesis for further clinical applications.

Keywords: Androgen receptors, Muscles of mastication, Temporomandibular dysfunction, Aging, Estrogen
Abbreviations: MM-Muscles of Mastication, AR-Anti-Androgen Receptor

Introduction

Muscles of Mastication (MM) dysfunctions is commonly associated with myofascial pain and it is more common in women (aged fifteen - fifty years) [1]. It was suggested that it is caused by gonado-corticoids and gonadal steroid fluctuations during menstrual cycle [2]. Other reports mentioned that women receiving contraceptive pills may suffer from MM dysfunctions which were attributed to the presence of estrogen receptors in either MM or supplying nerve fibers modulating the encoding of noxious stimuli, while many reports attributed the MM dysfunction to the presence of androgen receptors MM [3-5]. It was reported that testosterone modulates the trigeminal nerve fibers development together with digastric muscle reflexes [6]. Therefore, during the menstrual cycle, the fluctuation of sex hormones levels may cause MM dysfunction and injury [7]. It is also noteworthy that age is considered another parameter affecting MM [8].

Testosterone was used as a protective agent to MM in men aged sixty years in various clinical studies [9]. It was mentioned that sex hormones play a role in MM dysfunction [10]. But, for the best of our knowledge, number of sex hormones receptors in addition to the aging effect on such receptors are scanty. Therefore, this study was designed to shed the light on sex hormones (especially testosterone and estrogen) receptors expression in MM of both genders and on the effect of age on such receptors.

Materials and Methods

Chemicals

All chemicals and reagents, unless otherwise specified, were purchased from Sigma-Aldrich Chemical Co. (Missouri, USA).

Animals

Eighty albino wistar rats were used, Average age 12-24 months. Animals were individually housed. Free access to food and water was allowed. 12 light/dark cycles was kept. By the help of air conditions, the temperature was kept 25ºC (in accordance to national and institutional guidelines).

Experimental design

Rats were equally divided into four groups (n=20). Group-12F composed of 12-months female rats. Group-12M composed of 12-months male rats. Group-24F composed of 24-months female rats. Group-24M composed of 24-months male rats. As per protocol described by Marcondes et al. [11], estrous cycle phases were determined to by analyzing vaginal secretions daily for fourteen days to ensure the presence of proestrus phase (only female rats with proestrus phases were included in the current study).

Rats were allowed to acclimatize for two weeks in the animal house before scarification (by intraperitoneally injection of xylazine (single dose, 15 mg/kg) and ketamine (single dose, 30 mg/kg) (Bio-Veta®, USA) and MM dissection (masseter, temporalis, pterygoid, digastric muscles) [12].

Histopathological examination

Hematoxylin and eosin staining was done in accordance to Feldman and Wolfe [13] protocol. Briefly, fresh muscles tissue was cut into 1cm^3 immediately after extraction from the rats. It was placed in fixative 4% formalin and left for 48 hours then placed in tissue processing cassettes. By help of ascending grades of alcohol, tissue is dehydrated to remove water and formalin traces from tissue then immersed in xylene to remove alcohol and facilitate paraffin wax infiltration into the muscles tissue. Cassettes were placed on warm plates then tissue was removed and immersed in paraffin blocks. After paraffin solidification, the blocks were cut into 5 µm thick sections by using manually operated rotary microtome CUT 4050 (4050F, R) (Microtec Laborgeräte GMBH, Germany). Tissue sections were placed on glass microscope slides, rehydrated, stained with hematoxylin (stains nuclei in blue) for 10 minutes and eosin (stains cytoplasm in red) for 10 seconds. The stained tissue sections were dehydrated again by ascending grades of alcohol for 10 minutes than covered by coverslip. Histopathological examinations were performed by two expert histopathologists blinded to our study (ten overlapped fields per section) then the images were analyzed by Image J. 1.24 v. software (Volumetry®, USA)

Immunohistochemistry examinations

Immunohistochemistry was done in accordance to Ward and Rehg [14] protocol. Briefly, paraffin embedded tissue sections were sliced (5 µm thick) and mounted to charged slides. Sections were deparaffinized and rehydrated by descending grades of alcohol. Endogenous peroxidase activity was quenched by placing the tissue sections in 3% hydrogen peroxide for 10 minutes. 200 µl of diluted 1ry antibody [anti-estrogen receptor-α (ER-α) antibody, dilution 1:1000; anti-estrogen receptor-β (ER-β) antibody, dilution 1:1000; Anti-Androgen Receptor (AR) antibody (dilution 1:500), (ABCAM®, USA)] were mounted to the tissue. Slides were incubated overnight at 4°C in a humidified chamber. In next morning, slides were washed by wash buffer for 3 minutes then covered with 2 drops of Signal Stain Boost Detection Reagent followed by incubation at room temperature in humidified chamber for 30 minutes. 200 µl of Signal Stain® DAB (Biocompare, USA) were applied to each section. After staining, slides were immersed in distilled water then counterstained with haematoxylin to stain nuclei in blue for better visualization. Coverslips were applied, examinations were performed by to expert histopathologists blinded to our study scoring was done as follows:
0=negative, 1=weak, 2=mild, 3=moderate, 4=strong reaction. Ten fields per section were analyzed by Image J 1.24 v. software.

Statistical analysis

Statistical Package for Social Sciences (SPSS) software, 20 V. (SPSS Inc., USA) was used for data analysis. The statistical significance of differences between groups was validated using One-Way Analysis of Variance (ANOVA). Post hoc Tukey-Kramer test was used for groups' comparison. Data were expressed in mean ± Standard Deviation (SD) and probability value was considered significant if <0.05.

Results

Effect of age and sex on masticatory muscles ultrastructure

Light microscopy examination of rat' MM tissue sections stained with H and E showed normal histological architecture of different muscles of mastication in both group-12F and group-12M. Myofilaments appeared with neat arrangement with peripherally located nuclei. Group-24F and group-24M showed areas of muscle atrophy appeared in the form of wavy arranged myofilaments, hyper eosinophilic sarcoplasm, crowded nuclei with inflammatory cells infiltrations (**Figure 1**).

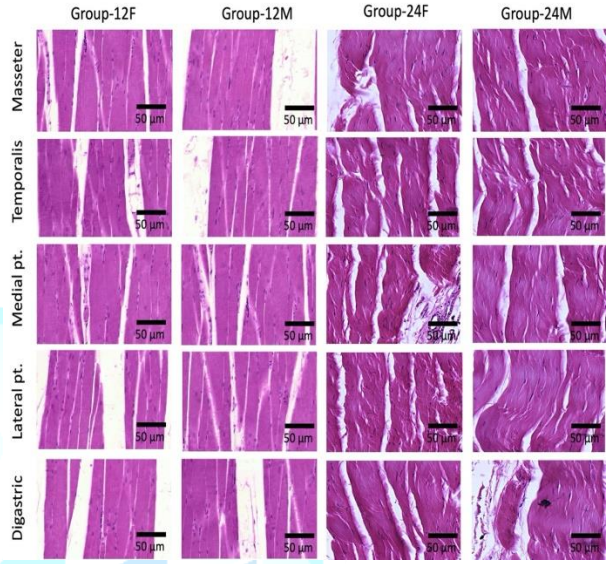

Figure 1: Photomicrograph of rats' muscles of mastication stained with hematoxylin and eosin (X 1000), (n=20). Normal histological architecture of different muscles of mastication is noticed in both group-12F and group-12M. Myofilaments are arranged normally with peripheral nuclei. Group-24F and group-24M show areas of muscle atrophy, wavy arranged myofilaments and inflammatory cells infiltrations.

Effect of age and sex on ER-α and ER- β receptors

Light microscopy examination of rat' MM tissue sections stained with anti-ER-α and ER-β antibodies showed low expression in different groups and muscles (**Figures 2-4**).

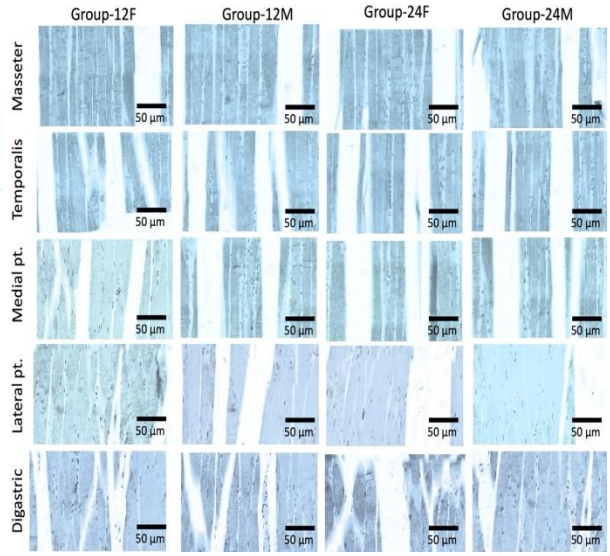

Figure 2: Photomicrograph of rats' muscles of mastication stained with anti-ER-α antibodies (X 1000), (n=20). Different groups and muscles show low expression of ER-α receptors.

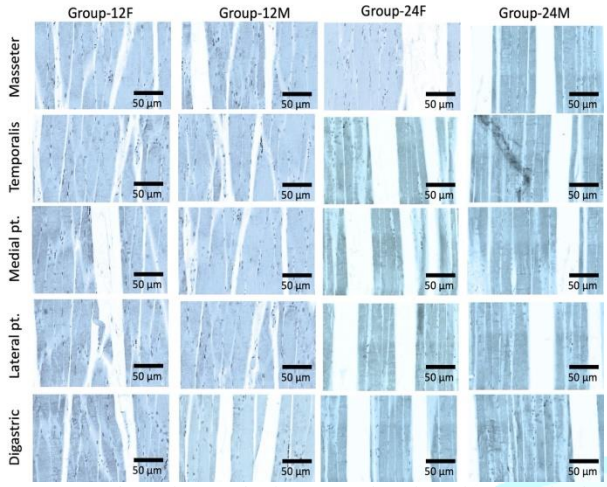

Figure 3: Photomicrograph of rats' muscles of mastication stained with anti-ER-β antibodies (X 1000), (n=20). Different groups and muscles show low expression of ER-β receptors.

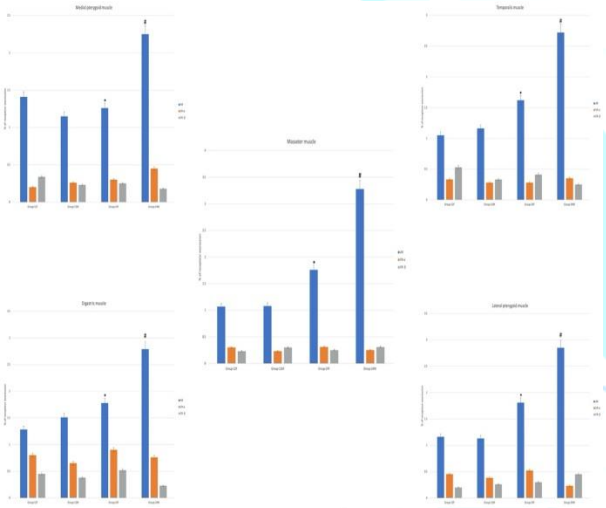

Figure 4: ER-α, ER- β and AR immunoreactivity in muscle of mastication. In group-24M, androgen receptor expression is highest in the masseter muscle. Androgen receptor expression in the masseter muscle is statistically significant ($p<0.05$) higher than digastric muscle. Non-significant differences are present between other muscles. ER-α, ER- β receptors expressions are very low in different groups and muscles. The statistical significance of differences between groups was validated using One-Way Analysis of Variance (ANOVA). Post hoc Tukey-Kramer test was used for groups comparison. Probability value was considered significant if $<0.05^{*}$. Statistically significant ($p<0.05$) difference in comparison to group-12F. #Statistically significant ($p<0.05$) difference in comparison to group-12M. Data are presented as mean ± SD, (n=20).

Effect of age and sex on AR receptors

Light microscopy examination of rat' MM tissue sections stained with anti-AR antibodies showed significant AR expression group-24M (especially in masseter muscle) if compared to other groups. In addition, group-24M showed that AR expression in the masseter muscle was significantly higher than if compared to digastric muscle, there were non-significant differences between other muscles of mastication (**Figures 4-5).**

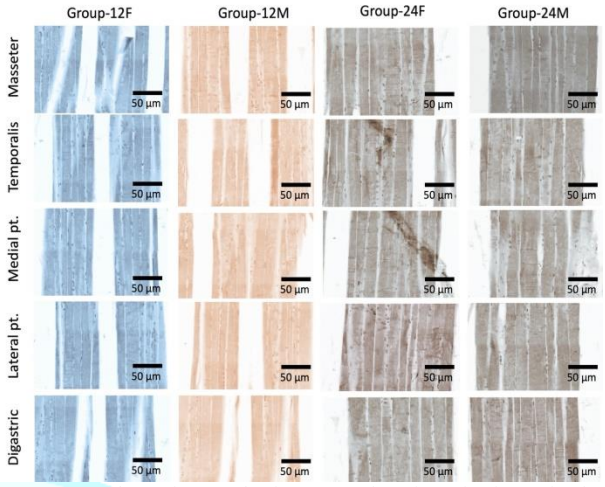

Figure 5: Photomicrograph of rats' muscles of mastication stained with anti-AR antibodies (X 1000), (n=20). Significant difference of AR expression in group-24M (especially in masseter muscle) can be noticed.

Discussion

Wang et al. [7] reported that dysfunctions of mastication muscles are presented with muscle pain and difficulties in mastication, Widmer et al. [5] attributed the age-related difficulties in speech and swallowing to MM dysfunctions which becomes more frequent in women (aged fifteen - fifty years) [15]. Current study results showed that androgen receptors located in MM may guard against age-related dysfunctions due to activation of anabolic pathway which results from its expression which could explain the vulnerability of females to these muscle related dysfunctions which comes in agreement with Iacovides et al. [15] who attributed the abundant presence of type II muscle fibers (fast-twitch) (essential for mastication movement) in masseter muscle to the activated expression of androgen receptor [12,13,16].

Shi et al. [17] reported that masseter muscle atrophy may cause Temporomandibular Joint (TMJ) movement disorder which depends primarily on the latter fibers. Baig et al. [18] reported that sustained androgen receptors expression with age helps in the maintenance of capillary network density in muscles. Iacovides et al. [15] reported the role of androgen receptors in increasing the mass of muscles (by enhancing type I and type II fibers growth) which results in muscular trophism and structural maintenance and linked between the short contraction intervals in the male rabbits' masseter muscle and the activated androgen receptors which sheds the light on the role played by these receptors in enhancing the physiological properties of MM as well which delay muscle fatigue resulting in less MM dysfunctions [14].

The present study showed that androgen receptor expression was highest in the masseter muscle of group-24M which may compensate to the age-related reduction in circulating testosterone which comes in agreement with Wilson et al. [19] who reported that serum testosterone levels drops to 25% of its normal value in old rats aged two years. Circulating Serum Testosterone (ST) is bound to Sex Hormone-Binding Globulin (SHBG) [affecting hormonal distribution and hence its biological activity] the latter functions and levels may be affected in malnutrition and obesity [20]. English and Widmer [21] reported the age-related increased activity of 5α-reductase enzyme (5α-R) in the prostatic acinar epithelium, which facilitate the irreversible conversion of testosterone into dihydrotestosterone (DHT), the latter has a more potent agonist effect on androgen

receptors. Some medications such as finasteride (proscar®), may reduce 5α-R activity in addition to androgen receptor affinity to ST.

Ekenros et al. [16] reported the anti-oxidant and anti-inflammatory potential of androgens which may also hinder age related MM-dysfunctions. ST is also crucial for the maturation of digastric muscle reflexes through increasing the neuronal density in the caudal part of trigeminal nerve nucleus extending through brain stem reflecting the protective role of testosterone against temporomandibular muscles dysfunction [18]. Many studies were done to explore the effect of testosterone on temporomandibular joint structural and functional properties and to identify the reasons of high prevalence of females to temporomandibular dysfunctions [13]. The androgen receptors in masticatory muscles could be considered the line of defense.

In conclusion, the present study sheds the light on the age-related increased expression of androgen receptors in male albino Wistar rats which could protect against temporomandibular muscles dysfunctions. Further studies are needed to evaluate this hypothesis for further clinical applications.

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