Original Article

Evaluation Of Azilsartan Transdermal Hydrogel In Volumetric Muscle Loss Injury In A Rat Model

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DOI: 10.47750/pnr.2022.13.S05.389

Abstract

A volumetric muscle loss (VML) is the loss of skeletal muscle with associated functional impairment, also known as traumatic or surgical muscle loss. Major muscle tissue loss occurs in personnel on battlefields who have suffered serious injuries. As a result of lost satellite cells, matrix degradation, and persistent upregulation of proinflammatory markers, the muscle cannot regenerate or achieve proper function. So, in the present study, we formulated the azilsartan hydrogel (0.5 and 1%) and further investigated the effect of the azilsartan (AZL) hydrogel in the VML rat model. For that, the volumetric muscle loss was done in the gastrocnemius muscle of 24 rats. The animals were divided into 5 groups: uninjured, VML, VML+ Ethanol, VML+AZL (0.5%) and VML+AZL (1%). The hydrogels were applied daily for 28 days at the injury site. Our results showed that AZL (1%) improved the gastrocnemius weight and their weight-to-length ratio. Also, the oxidative stress and antioxidant levels in skeletal muscle of VML were restored. In conclusion, these results showed that azilsartan hydrogel is effective in volumetric muscle loss induced in rats and could be taken as a therapeutic strategy.

Keywords: Volumetric muscle loss, Azilsartan, Hydrogel, Gastrocnemius, Oxidative stress.

INTRODUCTION

Traumatic muscle loss during battlefield or surgery is the primary reason for Volumetric Muscle Loss (VML) [1]. VML results in 20% or more muscle mass loss, inhibiting the self-repair pathway and causing significant functional impairment [2]. It damages the injury site cells and injures the nearby tissues that include nerves, bones, tendons, and blood vessels. An estimation showed that there is ~250,000 VML injury per year on the battlefield in the US [3]. 8% of medevac cases got handicapped due to VML injury. In a lifetime, $340,000-440,000 is invested by a disabled person due to VML injury [3]. VML injury results in the loss of regenerative cells such as satellite cells, the basal lamina, and fibroadipogenic progenitors [4].

Hydrogels have numerous advantages like they have a particle size within the nanometric range, follow the non-Newtonian pseudo plastic flow, having pH of acidic range, high bio adhesive property [5–8]. One of the main advantages of using hydrogel is their applicability *i.e.* easily applicable, and non-greasy. Azilsartan is an Angiotensin Receptor Blocker (ARB) highly selective for the AT1R and used to treat hypertension [9,10]. Lastra *et al.* showed that azilsartan improves insulin resistance and glucose transport activity in skeletal muscle *via* the Akt/AS160 signalling in the gastrocnemius muscle and soleus muscle [10]. Also, azilsartan significantly ameliorated the cardiac fibrosis in abdominal cardiac constriction (AAC) [11]. One interesting finding in the mice model of contusion injury showed that losartan shows muscle regeneration and improves muscle healing [12] and is proposed to use along with muscle-derived stem cells to treat skeletal muscle injuries. In the present study, we prepared azilsartan hydrogels as an appropriate treatment for VML-induced muscle injury. We have chosen the 1% Carbopol 940 as reported effective in loading in ARB drugs [13] due to its good adhesive property, consistency, nonirritant, and swelling volume [14–16].

MATERIALS AND METHODS

Materials

Azilsartan medoxomil was gifted by Honour lab limited, Telangana, India. All the chemicals used were purchased from Himedia and Sigma until unless specified. All solvents used in the present study were of analytical grade. Propylene glycol was purchased from Changshu Hongsheng Fine chemical Co. Limited, India.

Preparation of azilsartan hydrogel

1% Carbopol 940 was dispersed in 100 ml distilled water and left for 24 hours for swelling of Carbopol. Subsequently 0.1%, 0.5% and 1% of azilsartan was dissolved in 15% ethanol separately for 3 different formulations. Ethanol helps in the dissolution of the drug and enhances permeability. This solution was added slowly in Carbopol dispersion. To obtain a pH of 6.4-7.0 triethanolamine was added. 15% propylene glycol was added. It acts as a permeability enhancer and preservative (TABLE I) [13].

TABLE I COMPOSITION OF AZILSARTAN GEL (% W/W)

Ingredients	AZL (0.1%)	AZL (0.5%)	AZL (1%)
Azilsartan Medoomil	0.1	0.5	1
Carbopol 940	1	1	1
Ethanol	15	15	15
Propylene glycol	10	10	10
TEA	0.5	0.5	0.5
Distilled water	73.4	73	72.5

EVALUATION OF HYDROGELS

Homogeneity

Visual examination was done of prepared hydrogel gel for homogeneity [19].

Grittiness

The light microscope was used to evaluate the presence of particulate matter in the formulation [19].

Extrudability study

10 g gels were filled in aluminum collapsible tubes. Pressure for 10 seconds was applied to aluminum collapsible tubes to get gel extruded and form a 0.5 cm ribbon. Then weight in grams of extruded ribbon was noted [19].

Measurement of pH

1 g of the formulation was dispersed in 100ml distilled water and stored for 2 hrs. Then, the pH value was taken in triplicate. [19].

Viscosity study

Brookfield viscometer (Labtronics, Model LT- 730) was used. Spindle no. 4 was used at 60 rpm to measure the viscosity. Dial reading was noted [19].

Spreadability

A 2 cm diameter circle was drawn on a glass plate. 0.5g gel was placed in that circle and at the top, another glass plate was placed. For 5 minutes 500g was placed on the top plate. With its pressure gel spreads and the increased diameter was noted [18].

Permeation study

Jacketed modified diffusion cell with a diffusion area of 1.87cm^2 and receptor cell volume of 20 ml was used. Receptor compartment was filled with phosphate buffer of pH 7.4 and 2gm of gel was loaded in the donor compartment. The temperature of the receiver compartment was maintained at 37± 0.5°C by using a stirrer. Sampling was performed at intervals of 0.5, 1, 2, 3, 6, 12, and 24 hrs. The same amount of fresh phosphate buffer was added again to the receiver compartment. Drug content was analyzed by using UV spectrophotometer at 249 nm [20] .

In vivo study

All animal experiments were approved by the Committee for Control and Supervision of Experiments on Animal (CPCSEA) and under the regulation of the Institutional Animal Ethical Committee (IAEC). Female Wistar rats (approx. 120-140g) were used in this work. Animals were kept at a controlled temperature and humidity. A control light-dark cycle of 12hrs was provided to animals. Water and Standard laboratory feed were provided *ad libitum.* Animals were acclimatized to experimental conditions for 1 week before initiation of the experiment.

Animal experimental design

The animals were divided into 5 experimental groups (six animals per group). Group I: Uninjured group wherein rats were kept under anesthesia without injury. Group II: Volumetric muscle loss (VML) group received injury but no treatment. Group III: VML – ETH (Ethanol) group that received topical application of ethanol gel. Group IV: VML-AZL (Azilsartan) (0.5%) group that received topical application of 0.5% Azilsartan hydrogel. Group V: VML–AZL (1%) group that received topical application of 1% Azilsartan hydrogel.

Induction of volumetric muscle loss injury

Animals were anesthetized before the experiment. Hairs were removed by an electrical clipper. A longitudinal skin incision was made in the right leg at the calf area. Gastrocnemius muscle was exposed, and a cut was made at 60 % of

the length, 75% of the width, and 50% of the thickness. Bleeding was controlled by simple compression. A cut portion of muscle was sutured with polydioxanone 7.0 wire and skin was sutured with 3.0 silk sutures (Ethicon) [21].

METHODS

Evaluation of muscle injury

Body weight variation and Gastrocnemius weight to length ratio

The Body weight of animals of all groups was recorded every one cycle completion and calculates at the end of the study by the digital weighing balance and find out the changes in body weight during the study period. The initial body weight at the start of the experiment was compared with the final body weight at the end of the experiment [1,22]. After sacrifice gastrocnemius muscle was isolated and weight to length ratio was calculated in each group [23].

Injury width

Injury width on muscle was measured with a vernier caliper on the day of injury. At the injury site formulation was applied daily. After sacrifice again muscle injury width was measured using Vernier Caliper [24].

Injury area

An injury site image on 0 days was taken and with the help of Image J injury area was measured. After sacrifice again muscle injury site images were taken, and the remaining injury area was measured using Image J [25].

BIOCHEMICAL ESTIMATION

Superoxide dismutase estimation (SOD)

100 µl gastrocnemius muscle homogenate was added to the Tris HCl buffer (pH-8.5) and the final volume was adjusted up to 3ml with the same buffer. Next, 25 µl of pyrogallol was added and a change in absorbance was recorded at 420 nm at the one-minute interval for 3 minutes. A Blank was prepared in which tissue homogenate was absent. the presence of SOD inhibits the increase in the absorbance at 420 nm after the addition of pyrogallol. The amount of SOD present in a sample was calculated according to the following equation [26,27].

Lipid peroxidation (MDA)

In brief, 1 ml of gastrocnemius homogenate was taken in a tube, in this 0.5 ml of 30% trichloroacetic acid and 0.5 ml of 0.8% thiobarbituric acid (TBA) were added and covered with an aluminium foil. The tubes were kept in a shaking water bath at 80°C for 30 minutes. After that, the tubes were cooled for 15 minutes and then centrifuged at 3000 rpm for 15 minutes. Absorbance was recorded spectrophotometrically at 540 nm against a blank in which a homogenate sample was absent. Lipid peroxidation was calculated from the standard curve using the 1, 1, 3, 3-tetramethoxy propane, and gastrocnemius MDA value was expressed as nM MDA/µg of protein. The amount of MDA present in a sample was calculated according to the following equation [26, 27].

Statistical analysis

Results were shown as the Mean ± Standard Error of the Mean (SEM) for each group. Statistical analysis was performed using Graph Prism pad (version7.05) statistical software. The significance of the difference between multiple comparisons was evaluated by using a one-way analysis of variance (ANOVA). In case ANOVA showed significant differences, *post-hoc* analysis was performed with Tukey's test. The value of $p < 0.05$ was considered to be statistically significant.

RESULTS:

Evaluation parameters of Azilsartan hydrogel formulations

Homogeneity, extrudability, and spreadability were excellent in all formulations. No grittiness was observed in any formulation. The weight in grams of extruded ribbon during extrudability test was 0.058 gm, 0.051 gm, 0.063 gm of AZL (0.1%), AZL (0.5%) and AZL (1%) respectively. Spreadability was found 280%, 275%, and 220% of AZL (0.1%), AZL (0.5%) and AZL (1%) respectively. Viscosity was increased as the percentage of drugs increased from AZL (0.1%) to AZL (1%). pH was found to be 7.48, 7.50 and 7.55 of AZL (0.1%), AZL (0.5%) and AZL (1%) respectively as shown in TABLE II.

TABLE II EVALUATION PARAMETERS OF AZILSARTAN HYDROGEL FORMULATIONS

S.N.	Formulation code	Visual appearance	Homogeneity	Grittiness	Extrudability	Spreadability	pH	Viscosity (mPa.s)
1.	AZL 0.1 %	Thick, white Translucent	+++	--	+++	+++	7.48±0.06	7297.33 ± 1.52
2.	AZL 0.5 %	Thick, White, opaque	+++	--	+++	+++	7.50±0.01	73299 ± 2.64
3.	AZL 1 %	Thick, White, opaque	+++	--	+++	+++	7.55±0.01	73698.67 ±0.57

Excellent $^{+++}$, Good $^{++}$, Satisfactory $^{+}$, No skin irritation and grittiness^{--}

Evaluation of *ex-vivo* permeability of Azilsartan hydrogel formulations

AZL (0.1%) had shown best flux in comparison to AZL (0.5%) and AZL (1%). In the same pattern AZL (0.1%) had shown best, AZL (0.5%) had shown good, and AZL (1%) showed poor permeability coefficient as shown in Table III.

TABLE III EVALUATION OF *EX-VIVO* PERMEABILITY OF AZILSARTAN HYDROGEL FORMULATIONS

S.N.	Formulation	Flux (mg/cm^2/hr)	Permeability Coefficient (cm/hr)
1.	**AZL 0.1 %**	34.22 ± 1.42	2.54 ± 1.10
2.	**AZL 0.5 %**	34.79 ± 3.91	2.67 ± 1.52
3.	**AZR 1 %**	25.54 ± 7.54	2.04 ± 1.46

In vivo results of Azilsartan hydrogel formulation on volumetric muscle loss injury

Effect of Azilsartan (AZL) hydrogel on body weight and percentage weight variation in the VML model

28-days treatment of 0.5 and 1% azilsartan (AZL) hydrogel in VML rat model showed no statistically significant changes in body weight when compared with VML group, however, there was a significant reduction in body weight of the VML group as compared to the control. These results showed that VML exhibits muscle injury and may impact body weight as shown in Figure 1 A.

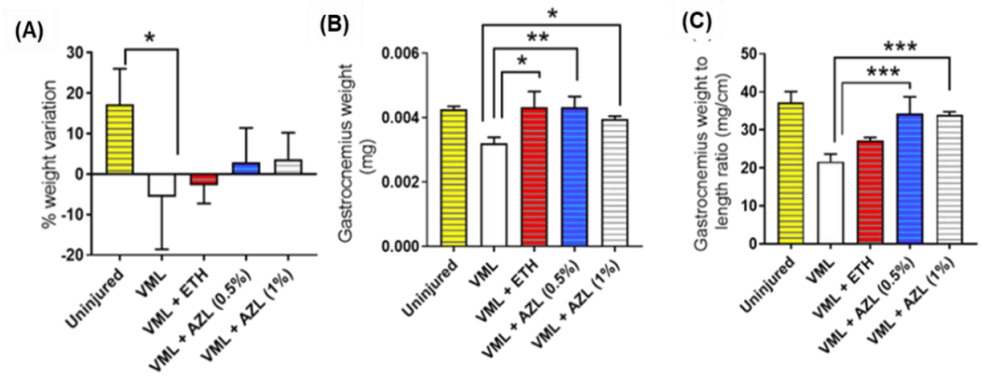

Figure 1: Effect of 0.5 and 1% Azilsartan hydrogel on (A) Percentage weight variation in VML model (B) Gastrocnemius muscle weight and (C) Gastrocnemius weight length ratio in VML model. All the values are expressed as mean ± SEM (n=6). ***p<0.0001, **p<0.001 and*p<0.05. Statistical analysis was done by one way ANOVA followed by Tukey's multiple comparison tests.

Effect of Azilsartan hydrogel on gastrocnemius muscle (GN) weight and gastrocnemius weight length ratio in VML model

Next, we assessed the GN muscle weight and its weight length ratio. Results showed both 0.5 and 1% azilsartan hydrogel treatment showed a significant increase in GN weight and weight length ratio in the VML group as compared to VML alone group. That showed that azilsartan hydrogel showed efficacy in muscle injury as shown in Figure 1 B- C.

Effect of Azilsartan hydrogel on gastrocnemius injury area and injury width in VML model

Further, to test the effectiveness of azilsartan hydrogel, the injury area on the 0 and 29th days were compared. Results showed that the area of injury gets statistically diminished on the 29th day in VML + Ethanol (ETH), VML+ AZL (0.5%), and VML + AZL (1%) groups when compared with the 0 day of the VML group as shown in Figure 2 (A). Next, the injury width of the 0 day and 29th day was compared. It was found that there was no change in injury width in the 0 day and 29th day VML group. However, there was significant decrease in injury width in VML + AZL (0.5%) and VML + AZL (1%) group as compared to VML Figure 2 (B).

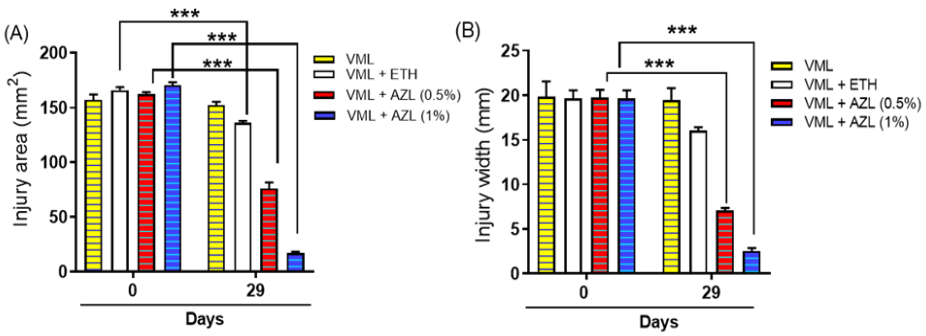

Figure 2: Effect of 0.5 and 1% Azilsartan hydrogel on (A) injury area (B) injury width of gastrocnemius muscle strength using tensiometry. All the values are expressed as mean ± SEM (n=6). ***p<0.0001. Statistical analysis was done by one-way ANOVA followed by Tukey's multiple comparison tests.

Effect of Azilsartan hydrogel on GN muscle antioxidant and oxidative stress levels in VML rat model

Next, we assessed the oxidative stress (Malonaldehyde, MDA) and antioxidant status (superoxide dismutase, SOD level) of GN muscle. Results showed that significant increase in SOD levels in VML + AZL (1%) as compared to VML. This depicts that azilsartan restored the antioxidant level. Further, the oxidative stress level was a significant decrease in VML + AZL as compared to AZL (Figure 3 A-B).

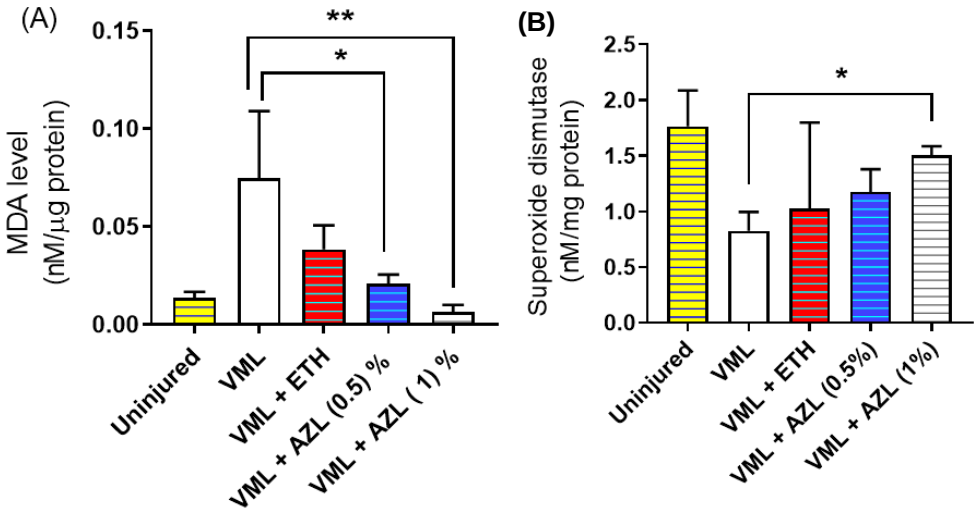

Figure 3: Effect of Azilsartan hydrogel on oxidative stress (A) MDA and antioxidant (B) Superoxide dismutase (SOD) level in gastrocnemius muscle of VML rat model. All the values are expressed as mean±SEM (n=6). **p<0.001 and *p<0.05. Statistical analysis was done by one-way ANOVA followed by Tukey's multiple comparison tests.

DISCUSSION

A complete recovery or repair of muscle loss in VML patients is currently not possible with current drugs. The global cost of VML's impact on health economics is rising quickly. Therefore, present study investigates the effectiveness of transdermal hydrogel of azilsartan in volumetric muscle loss injury. We had formulated three doses of azilsartan hydrogels *i.e.*, low, medium and high having percentages of 0.1%, 0.5%, and 1% Carbopol. Results of the present study showed that azilsartan hydrogel possessed excellent homogeneity, smooth texture, no grittiness, good spreadability, pH in the neutral range, and extrudability. Also, the *ex-vivo* drug release that showed a good permeability coefficient in AZL 0.5% and 1% were having the same range of permeability coefficient. *In vivo* results showed that azilsartan significantly improved the weight of gastrocnemius muscle in VML treated group. This result was further validated by the gastrocnemius weight to length ratio that showed significant improvement in VML+ AZL (0.5%) and VML + AZL (1%) groups. Next, compared the injury area on the 0 day to the 29th day and showed less difference in the VML group but after treatment, there is a significant decrease in the injury area of VML + ETH, VML+ AZL (0.5%), VML + AZL (1%) groups. The results were further supported by the same class of medication, 1% valsartan gel, which demonstrated a significant improvement in the mice's tensile strength and closure time [28]. They also showed a significant decrease in wound area after treatment. Next, a significant decrease in injury width after treatment further supports our hypothesis that azilsartan hydrogel is effective in VML injury. Angiotensin receptor blockers (ARBs) and angiotensin converting enzyme inhibitors (ACEIs) local application has recently been shown to reduce scarring by decreasing the expression of fibrosis markers and transforming growth factor-β [8]. Next, we measured the levels of different oxidative stress and antioxidants in gastrocnemius muscle tissue to determine whether azilsartan hydrogel was able to restore the muscle antioxidants status. There was a significant increase in superoxide levels in VML + AZL (1%) when compared with VML. Further, significant decrease in malonaldehyde levels in VML + AZL (0.5%) and VML + AZL (1%) as compared to VML. Overall. AZL (1%) showed better antioxidant effect then AZL (0.5%). Based on present findings our study concludes that azilsartan is more effective in the treatment of VML injury and AZL (1%) formulation is highly effective in the treatment of VML injury than AZL (0.5%).

CONCLUSIONS

This study provides valuable information to determine the effective topical dose of the formulation. It is achieved by comparing the different parameters and concluded AZL (1%) dose is more effective than AZL (0.5%) in volumetric muscle loss (VML) injury. These points aid in the future development of new topical formulations for VML injury.

AKNOWLEDGEMENT

We are highly thankful to availing instrumentation facilities to University Sophisticated Instrumentation Centre (USIC), Babasaheb Bhimrao Ambedkar University, Lucknow.

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