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THE ROLE OF *ACALYPHA INDICA* LINN EXTRACT ON EXPRESSION OF ACETYLCHOLINE RECEPTOR IN NEUROMUSCULAR JUNCTIONS WITH MYASTHENIA GRAVIS RAT MODEL

Rani Wardani Hakim[1], Desak Gede Budi Krisnamurti[1], Radiana Dhewayani Antarianto[2], Siti Farida[1], Erni Hernawati Purwaningsih[1], Jan Sudir Purba*[3]

[1]Department of Medical Pharmacy, Faculty of Medicine, Universitas Indonesia, Jalan Salemba Raya No. 6, Jakarta, 10430, Indonesia.

[2]Department of Histology, Faculty of Medicine, Universitas Indonesia, Jalan Salemba Raya No. 6, Jakarta, 10430, Indonesia.

[3]Department of Neurology, Dr. Ciptomangunkusumo General Hospital, Faculty of Medicine, Universitas Indonesia, Jalan Diponegoro No 71, Jakarta, 10430, Indonesia.

***Corresponding Author: Dr. Jan Sudir Purba MD, PhD**
Department of Neurology, Dr. Ciptomangunkusumo General Hospital, Faculty of Medicine, Universitas Indonesia, Jalan Diponegoro No 71, Jakarta, 10430, Indonesia.

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ABSTRACT

Objectives: Myasthenia gravis (MG) is one of the autoimmune disease for which the target autoantigen, the nicotinic acetylcholine receptor (AChR) of the neuromuscular junctions (NMJ). A number of molecules, including ion channels and other proteins at the neuromuscular junction, may be targeted by autoantibodies leading to abnormal neuromuscular transmission and produces symptomatic weakness that predominates in certain muscle groups and typically fluctuates in response to effort and rest. Treatment of MG involves the use of drugs standards for MG can only last for short time, and enormously high cost of treatment. By most ethnicities and cultures plants have been used as medicines throughout recorded human history. For this study of experimental autoimmune myasthenia gravis (EAMG) is used rocuronium, prostigmine and *Acalypha indica* Linn (*AI)* compared with expression of AchR. **Results:** Results of this study found that induction by rocuronium injection to animal model MG as competitive antagonist for acetylcholine receptor showed reduction of AChR expression in comparison to normal group. Preventive treatment with prostigmine showed increased AChR expression in comparison to MG induction group. Curative treatment with prostigmine showed decreased AChR expression in comparison to MG induction group and is lower than the normal group. Preventive treatment with Al extract showed comparable level of AChR expression as MG induction group. Curative treatment with Al extract showed decreased AChR expression in comparison to MG induction group as well as normal group. However, AChR expression by curative Al extract is at comparable level as curative prostigmine. **Conclusion:** Results of this study used prostigmine as a preventive therapy which afterwards given rocuronium, amount of AChR did not differ significantly. Likewise, AI given as a curative therapy is also not significantly different from the effect of prostigmine. As a conclution the use of AI as a preventive or curative therapy indicates that AI has an effect similar to the effect prostigmine which can prevent a decrease in AChR in rats induced by rocuronium. Our results clearly demonstrate Acalypha indica Linn as a promising candidate for the new drug for myasthenia gravis.

KEYWORDS: Myasthenia gravis; extract Acalypha indica Linn; Neuromuscular junction; expression of AChR.

INTRODUCTION

Myasthenia gravis (MG) is an antibody-mediated autoimmune disease that targets the neuromuscular junction (NMJ). How autoantibodies develop against acetylcholine (ACh) receptors is not yet known. Acetylcholine is the neurotransmitter used at the NMJ. It is the chemical that motor neurons of the nervous system release in order to activate muscles. Cholinergic nerve conduction to striated muscle is impaired by a mechanical blockage of the binding site by antibodies and, ultimately, by destruction of the binding site of the certain nerve receptors namely acetylcholine receptors (AChR) to function properly.[1,2] A reduction in the number of AChR results in a special situation of progressively reduced muscle strength with repeated use of the muscle and recovery of muscle strength following a period of rest. In patients with MG, the disease arises from a humoral auto-immune response directed against the muscle AChR.[1,2] Evidence from classical experiments indicates that anti-AChR antibodies are pathogenic, the main cause of weakness in MG, leading

to end-plate AChR loss, simplification of the postsynaptic membrane and derangement of neuromuscular transmission.[2] Confocal microscopy showed that delayed synapsing muscles are more severely affected than fast-synapsing muscles.[3] AChR antibodies cause loss of functional AChR at the NMJ, either by crosslinking the receptors leading to increased internalization (antigenic modulation), by activation of complement leading to focal lysis and morphological damage to the postsynaptic membrane, or by inhibiting the AChR's ion channel function.[4]

Patients with MG exhibit characteristic fatigable weakness of voluntary muscles including ocular, facial, oropharyngeal, limb and respiratory muscles.[1,6,7] Predominant symptoms are dysarthria, nasal voice, weakness of facial and limb muscles.[5,6,7] Weakness progressively affects bulbar muscles, which are the most severely affected during the course of pathology.[5,6] In some cases, only ocular muscles can be affected (Hain, 2004). Cardiac involvement in myasthenia gravis may take several forms, ranging from asymptomatic Electro cardiograph (ECG) changes to ventricular tachycardia, myocarditis, conduction disorders, heart failure and sudden death.[8]

Autoimmune MG is a relatively rare disease affecting approximately 20 per 100,000 people.[9] Based on 55 studies spanning 1950–2007, calculations of total MG incidence and prevalence, have yielded a pooled incidence rate (IR) of 5.3 per million person-years and a prevalence rate (PR) of 77.7 cases per million of the population [10]. Women are frequently more affected than men.[5,6] Marked heterogeneity and the varying quality of epidemiological studies, were, not surprisingly, notable factors influencing these estimations over so many years.[10] Nevertheless, it is well recognised that MG prevalence has been rising since the middle of the last century,[11] with improved recognition and diagnosis, medical and intensive care advances and patient longevity all playing a role,[10,11]

Treatment of MG involves the use of acetylcholinesterase inhibitors, immunosuppressive drugs, thymectomy, plasmapheresis, and the i.v. administration of human immunoglobulins can only last for short time, and enormously high cost of treatment. The needs and urgency for alternative treatment for myasthenia gravis has becoming more and more important.[12] Plants have been used as medicines throughout recorded human history by most, if not all, ethnicities and cultures. Modern science has devoted considerable research to characterizing the efficacy and mechanisms of action of many medicinal plants, but this remains an area of vast research potential. Studies with herbal plant in Indonesia were developed for the purpose of preventive and curative for MG. One of the therapeutic development for MG are using herbal ingredients of *Acalypha indica Linn (AI)*. In previous study the water extract of the roots of *AI* for animal models proved to have an effect therapy.[13,14] To know the effect extract of roots *AI* against the AChR and enzyme acetylcholinesterase on the NMJ in animal models NMJ MG, Spraque-Dawley (SD) rats were injected with rocuronium bromide. For his studies mechanisms of plant *AI* as therapies on MG done by assessing the AChR on the NMJ condition will be tested with immunohistochemistry.

MATERIALS AND METHODS

Method

Extraction process

Dry root of *AI* were collected from Depok and had been identified at Lembaga Ilmu Pengetahuan (LIPI, Indonesian Institute of Sciences) Bogor. These roots were dried and powdered. The powder was macerated by ethanol 70% for 24 hours. Subsequently, the residue was remacerated three times by the same solvent, and the extract was mixed into previous ones. The collected extract was then concentrated by rotary vacuum evaporator. The dose of *AI* in this study was 150 mg/kg/BW. The extract was dissolved in aquadest to get the appropriate dilution to administer orally just before the treatment exposure on each day.

Treatment

Animal involved in this experimental study were 35 *Spraque-Dawley* male rats, 8 week of age, 150-200 gram. The rats were adapted into the experimental cages at the room temperature of 24˚ C, for a week before and during treating. They were divided into six groups of experiment, randomly. Group control is healthy SD rats without any treatment designated as control negative or normal group. For this experiment used rocuronium, prostigmine and *AI* as compared with the expression of the AChR. Rocuronium is a muscle relaxants non-depolarising (competitive inhibitors) that bind to nicotinic-cholinergic receptors, but does not cause depolarization, blocking acetyl choline and occupied it, whereas prostigmine is a cholinesterase inhibitor used in the treatment of myasthenia gravis and to reverse the effects of muscle relaxants. Extract *AI* estimated can neutralize toxin caused by auto-immune and used as a therapy MG. Group control (normal) received CMC-Na Sol.1%.

The treatment divided into two set, *AI* as a curative treatment and *AI* as a preventive. As a curative treatment, rats divided into 3 groups. Rats in group rocuronium was injected with rocuronium intraperitoneal 10 mg/kg.BW as a control negative; group rocuronium + pros was injected first with rocuronium then after 10 minutes injected with prostigmine (as a positive control); and group rocu + *AI* was injected first with rocuronium then after 10 minutes was given *AI* extract.

As a preventive rats divided into 3 groups. Rats in group rocu was injected with rocuronium intraperitoneal 10 mg/kg.BW, group pros + rocu was injected first with prostigmine then after 10 minutes injected with

rocuronium; group *AI*+rocu was given *AI* extract 250 mg/kgBW first then after 30 minutes was injected with rocuronium. The treatment was given for three days, at the end of day 3, each rat was sacrificed under anesthesia. This study was performed from May 2016 to January 2017 at Faculty of Medicine Universitas Indonesia, Cipto Mangunkusumo Central Hospital (FKUI-RSCM), Histology Lab FKUI and Animal Lab National Institute of Health Research and Development Ministry of Health Republic of Indonesia. Protocol of this study has been approved by Ethical Committee Faculty of Medicine, Universitas Indonesia.

Histology specimen preparation and processing

After SD rats are sacrificed, the hind legs are removed and skin from the legs are detached from surrounding tissue using tissue pinset and scissors. Initially, hind leg muscle and bones are processed in bone de-calcifying solution for 3-5 days. Further optimation showed removal of skeletal muscle prior to de-calcifying solution yield shorter histology processing period and preservation of skeletal muscle size and tonus as freshly isolated specimen. Thus, isolation of Gastrocnemius muscle and the attached tibialis posterior muscle (skeletal muscle sample) are collected and underwent histology processing. Briefly, skeletal muscle samples are fixated in 4% formol saline for 24-48 hours. Subsequent dehydration techniques are performed by immersion of skeletal muscle sample in increased gradient alcohol concentration (70%-80%-90%-95%-100%) for 5-7 days. Clearing of skeletal muscle sample in Xylol solution is performed prior to molding into paraffin wax inside a cassette termed blocks. These blocks are sectioned using microtome with 5-6 µm thickness. The delicate paraffin embed section are spread on the water surface of warm waterbath (37^0C) and carefully mounted on top of histology slide. These slides are dried overnight in the oven and ready for subsequent staining.

Hematoxyllin eosin staining

Deparaffinization in Xylol 2x5 minutes. Wash slides in PBS 2x5 menit. Gradual rehydration with graded alcohol concentration: alcohol absolute – alcohol 90% - alcohol 80% - alcohol 70% - alcohol 50% each for 5 minutes. Incubation in hematoxyllin Harris solution for 15 minutes. Washed the slides in running tap water for 5 minutes, quick dip in acid-alcohol solution for 3-10 times. Washed the slides in running tap water for 10 minutes. Incubation in eosin solution for 2 minutes. Gradual dehydration with graded alcohol : alcohol 50%, alcohol 70%, alcohol 80%, alcohol 90%, absolute alcohol each for 5 minutes. Clearing with Xylol for 2x5 minutes. Embedded with Entelan and closed with cover glass. Stained histology slide is observed under light microscope and documented using Optilab. Integrity of the skeletal muscle sample and motor end plate structures are requirements for further immunohistochemistry procedure.

Immunohistochemistry Acetylcholine Receptor (AChR) of skeletal muscle

Immunohistochemistry of Acetylcholine receptor AChRα4 (sc-1772, Santa Cruz) is conducted for detection of the acetylcholine receptor α4 subunit of rat tissue in paraffin embedded sections. The secondary antibody and chromogen substrate all included in Goat Immunocruz staining system (sc 2023, Santa Cruz). Deparaffinization in xylol 2x5 minutes. Wash slides in PBS 2x5 menit. Gradual rehydration with graded alcohol concentration: absolute alcohol absolut – alcohol 90% - alcohol 80% - alcohol 70% - alcohol 50% each for 5 minutes. Wash slides with de-ionized water 2x5minutes. Blocking of endogenous peroxidase with H_2O_2 3% (Sigma) inside moist chamber incubation at room temperature for 20 minutes. Wash with de-ionized water 2x5 minutes. Blocking of non-specific avidin/biotin binding: the slides were covered with 1.5% donkey blocking serum and incubated inside moist chamber for 1 hour at room temperature. Wash slides with de-ionized water 2x5 minutes. Overnight incubation with primary antibody againsts AChRα4 and 1.5% donkey blocking serum for negative control inside the moist chamber. Moist chamber is placed in the refrigerator (4^0 C). Day 2, wash slides in PBS solution 2x5 minutes. Incubation of slides with biotinylated secondary antibody is performed for 1 hour in moist chamber at room temperature. Wash slides in PBS 2x5 minutes. Followed by streptavidin-conjugated-peroxidase incubation for 30 minutes. Wash slides in PBS 2x5 minutes and further DAB substrate addition for 1 minute. Nuclear counterstained with Hematoxylin Harris. Slide was observed under light microscope and documented with Optilab. Five high power fields (hpf, magnification 400x) are randomly selected from each slide. Areas of dark brown signals (Acetylcholine receptor positive) are counted in each hpf.

RESULTS

Histology of gastrocnemius muscle

Routine staining (HE staining) of skeletal muscle showed histology of long un-branched striated fibers with multiple nuclei at periphery of each fiber as depicted in figure 1. Orientation of the skeletal muscle is longitudinal section in oblique arrangement. In between the skeletal muscles hollow structure lined by endothelial cells or known as capillary structures are identified by the white arrows in figure 1. In this study, HE staining is the pre-requisite step before specific identification of post synaptic acetylcholine receptor expression. Integrity of the skeletal muscle tissue is shown in figure 1 as the arrangement of the fibers are saline well and characteristic of skeletal muscle is intact.

The distinctive structure of neuromuscular junction which is a structure of myelinated axon terminates at terminal buttons or pre-synaptic membrane is difficult to visualize using HE staining. Figure 1 indicated plausible location of neuromuscular junction. However it's not definitive. Specific location of motor end plate in this

study is the site of Acetylcholine receptor/s expression at skeletal muscle membrane. Identification of Acetylcholine receptor expression in the tissue is accomplished through immunohistochemistry method.

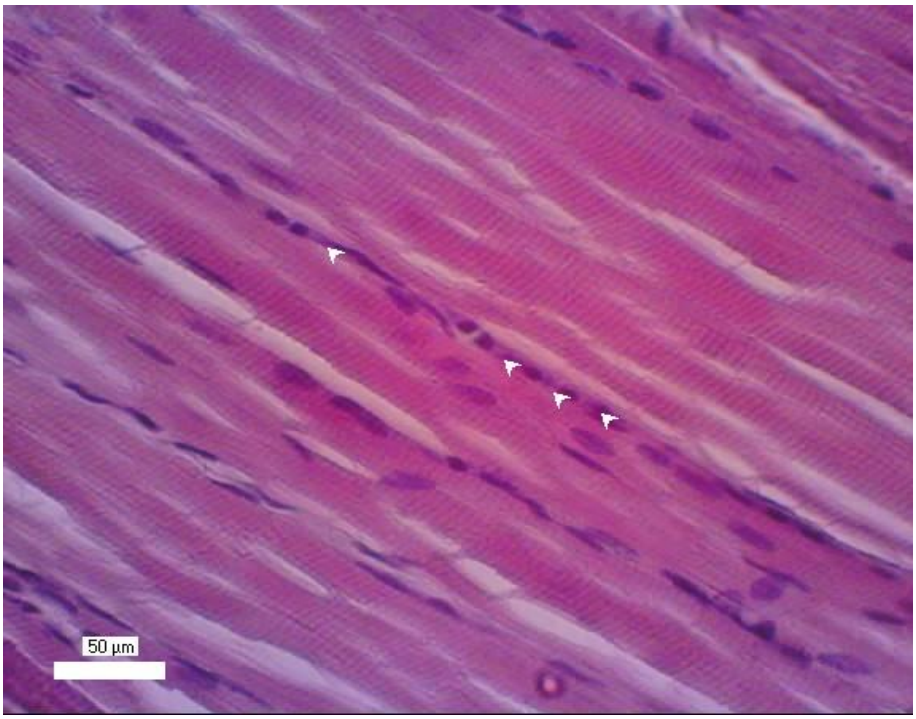

Figure 1. Histology of skeletal muscle in longitudinal section. HE staining. Magnification 400x. White arrows indicate endothelial cells lining a capillary.

Distribution of Acetylcholine receptor expression at skeletal muscle membrane

Negative expression of AChR is shown in figure 2A which is the negative control of immunohistochemistry AChR. The skeletal muscle membrane appeared in bluish striation with multiple nuclei at the periphery. Positive expression of AChR is shown in figure 2B as chocolate to dark chocolate aggregates circled in white on the surface of skeletal muscle membrane.

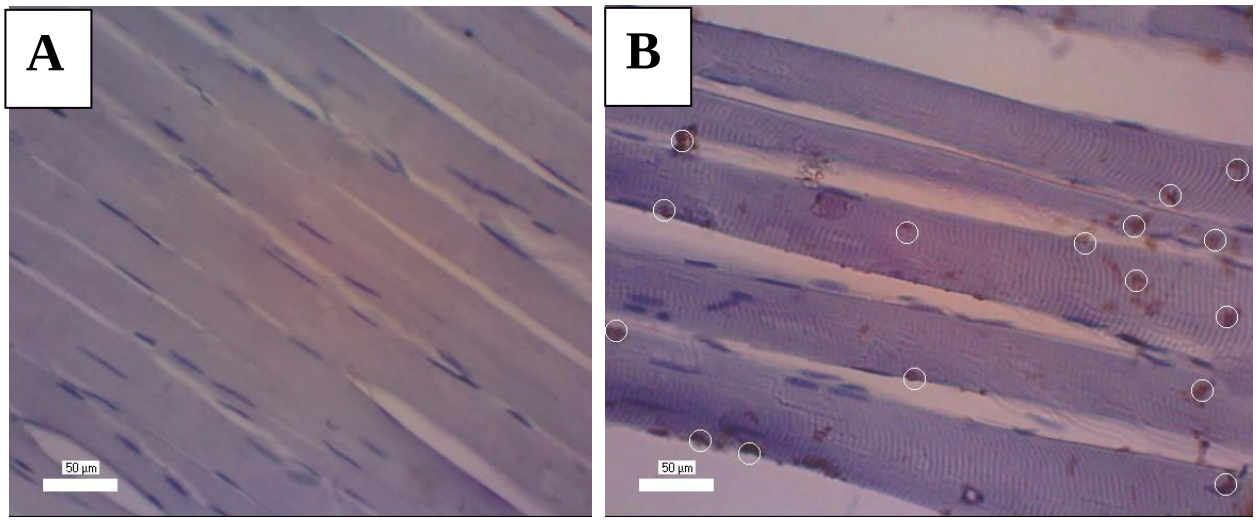

Figure 2: Immunohistochemistry of AChR in skeletal muscle. Negative control showed absent of AChR expression (Fig.2A). AChR expression in skeletal muscle positive reaction to DAB chromogen substrate which yield brown to dark brown colour (Fig.2B). AChR expression was distributed on the surface of skeletal muscle membrane or plasmalemma.

Data Analysis

Data analysis is descriptive analysis using Microsoft Excell 97 program. Number of acetylcholine receptor positive sites from each sample are mean of AChR from five hpf. Each group have in total 5 mean of AChR positive per hpf. Mean of each group is displayed in bar

graph and error bars are determined from standard error of mean by Excell chart program. Comparison between groups is described as comparison between mean and standard error of mean from each group.

Acetylcholine receptor expression comparison from five treatment group and normal group

Number of AChR from five hpf was counted (figure 2). Mean of five hpf from each subject in group 1 to 6 was calculated. From figure 3, induction of MG (group 1) showed reduction of AChR expression in comparison to normal group (group 6) with mean ±SD = 9.28±2.19 per hpf vs 11.4±6.76 per hpf. This signifies the MG induction by rocuronium injection as competitive antagonist for acetylcholine receptor.

Preventive treatment with prostigmine (group 2) showed increased AChR expression in comparison to MG induction group (group 1) with mean ±SD = 11.92±1.79 per hpf vs 9.28±2.19 per hpf, respectively. The increment of AChR expression by preventive prostigmine is at a comparable level with normal group (group 6) = 11.92±1.79 per hpf vs 11.4±6.76 per hpf, respectively.

Curative treatment with prostigmine (group 3) showed decreased AChR expression in comparison to MG induction group (group 1) with mean ±SD = 6.65±2.17 per hpf vs 9.28±2.19 per hpf, respectively. AChR expression by curative prostigmine is lower than the normal group (group 6) = 6.65±2.17 per hpf vs 11.4±6.76 per hpf, respectively.

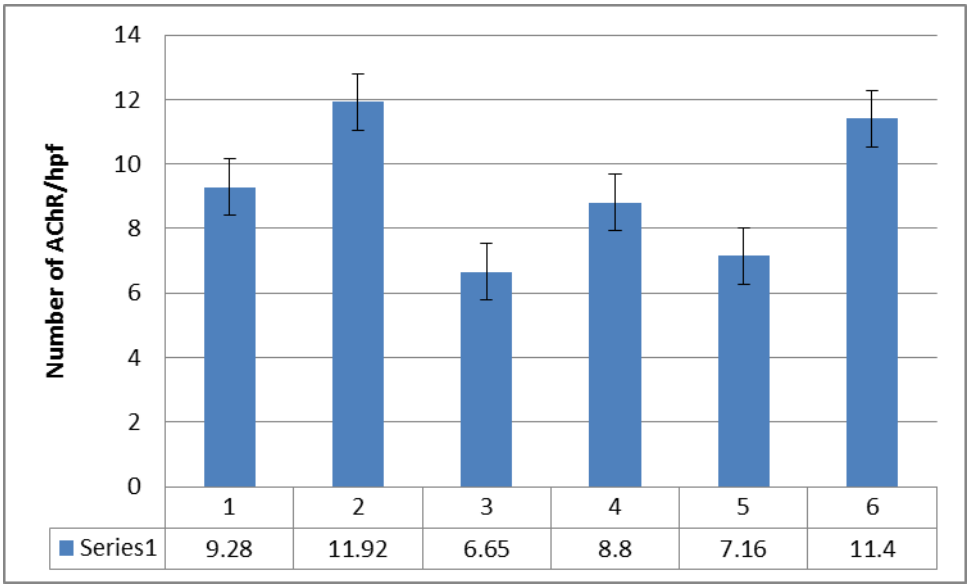

Figure 3: Number of Acetylcholine receptor per hpf. Bar indicate mean from total number of acetylcholine receptor in each hpf field from 5 subjects in group 1 to 6. The mean value of each group is shown in horizontal axis. Error bar indicate 5% value from mean of each bar.

Preventive treatment with *Al* extract (group 4) showed comparable level of AChR expression as MG induction group (group 1) with mean ± SD = 8.8±5.06 per hpf vs 9.28±2.19 per hpf, respectively. The decrease of AChR expression by preventive *Al* extract is lower than the normal group (group 6) = 8.8±5.06 per hpf vs 11.4±6.76 per hpf, respectively. This result is in contrast to preventive prostigmine which showed increment of AChR expression as comparable level as normal group.

Curative treatment with *Al* extract (group 5) showed decreased AChR expression in comparison to MG induction group (group 1) with mean ±SD = 7.16±0.83 per hpf vs 9.28±2.19 as well as normal group. However, AChR expression by curative *Al* extrct is at comparable level as curative prostigmine (group 3) with mean±SD = 7.16±0.83 per hpf vs 6.65±2.17 per hpf , respectively.

Histologic comparison of Acetylcholine receptor expression between groups

MG induction group showed distribution of AChR expression limited to several skeletal muscle fibers within one hpf (Figure 4 A), while in normal group all the skeletal muscle fibers in one hpf showed positive AChR expression (Figure 4 B). This histologic feature is predominant finding in hpf from subjects in group 1 and group 6.

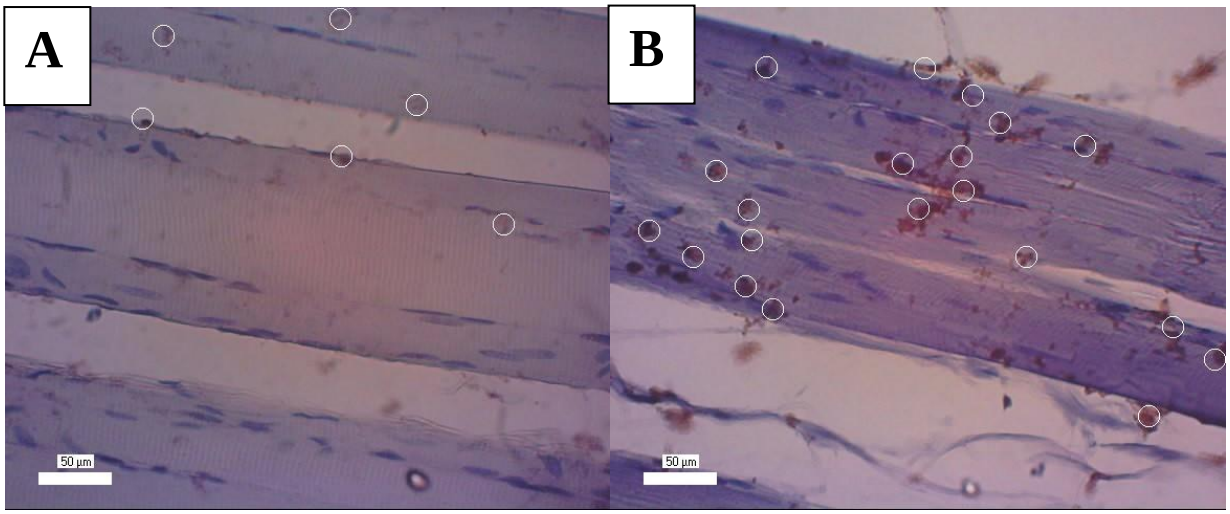

Figure 4. Representative image of AChR expression from group 1 (Fig.4A) and group 6 (Fig.4B). Magnification 400x. Immunohistochemistry of AChR. Representative image from group 1 showed several skeletal muscle fibers immunoreactivity with AChR antibody, brown to dark brown colour circled in white indicate AChR expression (Fig. 4A). Representative image from group 6 showed all skeletal muscle fibers immunoreactivity with AChR antibody, brown to dark brown colour circled in white indicate AChR expression (Fig. 4B).

Preventive prostigmine group showed distribution of AChR expression in almost all skeletal muscle fibers within one hpf (Figure 5 A), while in preventive *Al* extract group showed less AChR expression in one hpf (Figure 5 B). Distribution of AChR expression in group 2 and group 4 is highly variable. This histologic pattern is consistent in most of hpf from subjects in group 2 and group 4 with exception in variable hpf.

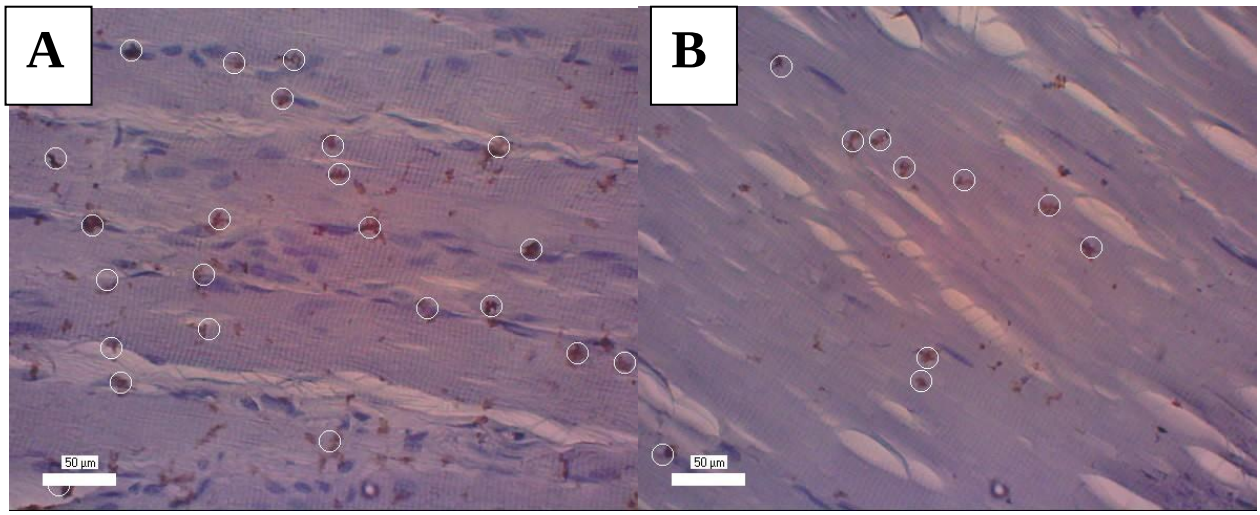

Figure 5: Representative image of AChR expression from group 2 preventive prostigmine (Fig.5A) and group 4 preventive *Al* extract (Fig.5B). Magnification 400x. Immunohistochemistry of AChR. Representative image from group 2 showed abundant skeletal muscle fibers immunoreactivity with AChR antibody, brown to dark brown color circled in white indicate AChR expression (Fig. 5A). Representative image from group 4 showed fewer skeletal muscle fibers immunoreactivity with AChR antibody, brown to dark brown color circled in white indicate AChR expression (Fig. 5B).

Curative prostigmine group showed AChR expression in a few skeletal muscle fibers within one hpf (Figure 6 A) and curative *AI* extract group showed comparable AChR expression in one hpf as curative prostigmine (Figure 6 B). Distribution of AChR expression in group 3 and group 5 is highly variable. This histologic pattern is consistent in some of hpf from subjects in group 3 and group 5 with exception of variable hpf. Both curative prostigmine and curative *Al* extract showed low AChR expression and distribution in comparison with MG induction control, preventive prostigmine, preventive *Al* extract and normal group.

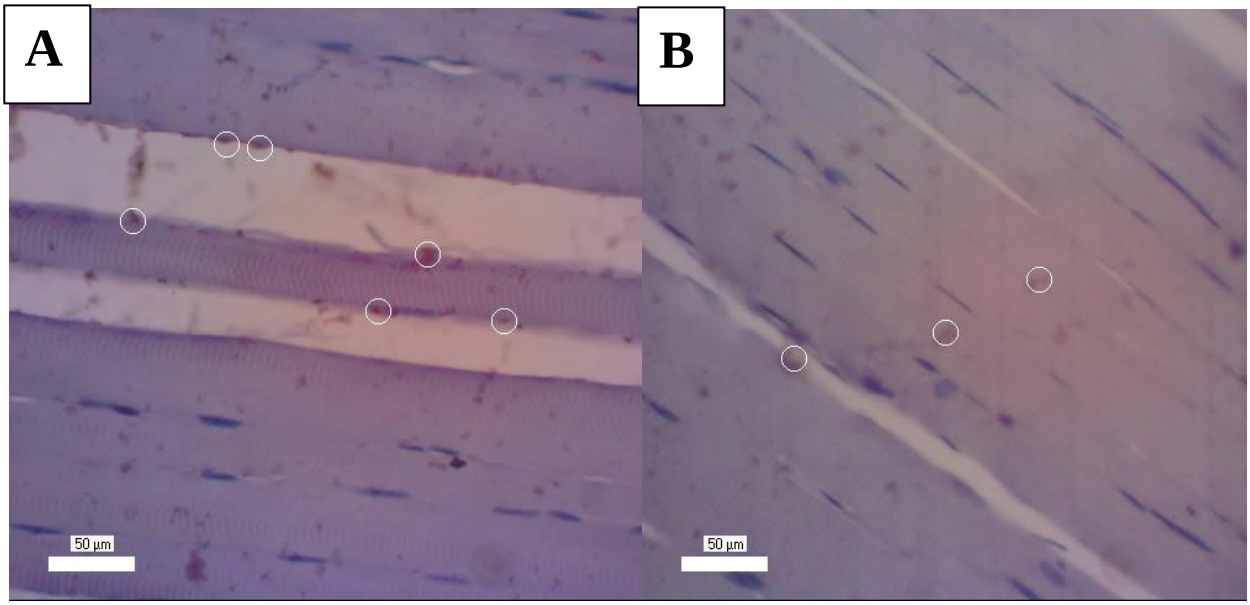

Figure 6: Representative image of AChR expression from group 3 curative prostigmine (Fig.6A) and group 5 curative *Al* extract (Fig.6B). Magnification 400x. Immunohistochemistry of AChR. Representative image from group 3 showed a few skeletal muscle fibers immunoreactivity with AChR antibody, brown to dark brown color circled in white indicate AChR expression (Fig. 6A). Representative image from group 5 showed fewer skeletal muscle fibers immunoreactivity with AChR antibody, brown to dark brown color circled in white indicate AChR expression (Fig. 6B).

DISCUSSION

Myasthenia gravis (MG) is a chronic autoimmune disease caused by the immune attack of the neuromuscular junction NMJ. The molecular organization of the NMJ is designed for optimal transmission of the signal from nerve to muscle (neuromuscular transmission), with nicotinic AChR clustered at high density on the postsynaptic muscle membrane.[15] In MG, the AChR is the main autoantigen, and the postsynaptic membrane of the NMJ is the target for antibody-induced damage. The antibodies cause loss of functional AChRs by cross-linking the receptors, leading to increased turnover of the AChR (antigenic modulation), by activating complement and leading to focal loss of the postsynaptic membrane folding, and/or by blocking the AChR ion channel.[4] Loss of functional AChRs compromises neuromuscular transmission, resulting in skeletal muscle weakness. Pathogenic anti-AChR antibodies are the primary cause of post-synaptic membrane dysfunction in approximately 85% of patients with autoimmune MG and the majority of these antibodies are directed against the α-subunit of the skeletal muscle AChR. Striated cardiac muscle can be also a target for immune attack manifesting as heart failure, arrhythmia, and sudden death.[16] A few studies were carried out to investigate cardiac involvement in patients with MG. In our previous study, it seems that on the basis of these results by using drugs rocuronium, prostigmine and *AI* is associated with heart rate on EAMG, curative therapy *AI* has the same effect with prostigmine.[14]

This research was conducted in SD rats by using rocuronium, prostigmine and *AI* as herbal therapy for MG to determine whether the root *AI* have a therapeutic effect as a binding antibody AChRs in animal models to have an effect therapies. The targets are postsynaptic proteins, mainly involving the skeletal muscle acetylcholine receptor (AChR) and the muscle-specific tyrosine kinase (MuSK).[17,18] It is suspected that *AI* has a similar effect to prostigmine. From the results of this study used prostigmine as a preventive therapy which afterwards given rocu, amount of AChR did not differ significantly. Likewise, *AI* given as a curative therapy is also not significantly different from the effect of prostigmine. As a conclution the use of *AI* as a preventive or curative therapy indicates that *AI* has an effect similar to the effect prostigmine which can prevent a decrease in AChR in rats induced by rocuronium. To know and learn more advanced study required.

CONCLUSION

From the results of this study can be concluded that the effect therapy of *Acalypha indica* Linn both as preventive and curative based on distribution of AChR expression, have not different from therapy of prostigmine. Our results clearly demonstrate *Acalypha indica* Linn as a promising candidate for the new drug for myasthenia gravis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

ACKNOWLEDGMENTS

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