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EARLY CO-ADMINISTRATION OF VITAMIN E ACETATE AND METHYLCOBALAMIN PREVENT PROGRESSION OF NEUROPATHIC COMPLICATION IN DIABETIC RATS

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ABSTRACT

Oxidative stress has been implicated in the eiteology of diabetic neuropathy (DN) and anti-oxidants have shown to ameliorate this condition. Previous studies have indicated that early treatment with vitamin E acetate (VE) and methylcobalamin (MCA) attenuated surgery induced neuropathy in rats. The aim of this study was to test the effect of early co-administration of VE (50 mg/kg) and MCA (500µg/kg) (VEM) on the development of DN in laboratory rats. Male Wistar rats were divided into the three treatment groups, sham (non-diabetic, vehicle treated), diabetic rats (vehicle treated; (DU), and diabetic rats which received VEM treatment (DVEM). Diabetes was induced by single injection of alloxan monohydrate (120 mg/kg). Body weights of all subjects were noted daily and serum glucose levels (SGL) were measured at regular intervals. Behavioural assessment of thermal hyperalgesia (TH) was performed using the hot plate test and warm water tail immersion test. Finally, motor nerve conduction velocities (MNCV) were measured on day 45. Significant enhancements in SGL were noted for DU rats and this remained throughout the experimental span. A corresponding reduction in latency times for withdrawal from thermal stimuli was noted for DU treated rats which was not the case for DVEM group of animals. In addition to its effect on lowering pain thresholds, daily VEM treatments showed significant improvement in MNCV compared to DU treated animals. These results indicate an ameliorating effect of VEM treatment in diabetic rats. The underlying mechanisms by which VEM acts needs further evaluation.

INTRODUCTION: One of the major concerns with uncontrolled diabetes is the development of microvascular complications such as neuropathy, cardiovascular, nephropathy, retinopathy and erectile dysfunction[1,2]. Among these complications, symptoms of diabetic neuropathy have been observed to emerge at the early stages[3]. These symptoms include hyperalgesia (exaggerated response to non-noxious stimuli) and allodynia (low threshold pain stimuli) have commonly been reported in diabetic patients [1, 2, 3, 4]. If untreated, these symptoms could progress into diabetic foot disorders, which in worst cases can even lead to amputations [1,4].

Common treatment strategies for DN include controlling diabetes and targeting painful symptoms associated with peripheral neuropathy [3, 4]. These symptoms have been managed clinically by using analgesics, tricyclic antidepressants and anti-convulsants [4].

However, this kind of management provides symptomatic relief rather than addressing the underlying pathophysiology of the DN [1]. Moreover, in many cases, only anti-nociception therapeutics has not been completely effective in managing the painful symptoms [1]. Therefore, there is a need to determine effective therapeutic strategies aimed at preventing microvascular complications associated with diabetes [1, 4]. Such strategies should apparently be aimed at correcting the pathophysiological and biochemical alterations associated with diabetes [2, 5].

Previous published literature indicates following molecular mechanisms implicated in hyperglycemia-mediated vascular damage. These includes the overproduction of advanced glycation end product, activation of mitogen activated protein kinases, enhancements in the polyol pathway flux and increased hexosamine pathway flux [1, 2]. All these cumulatively trigger the production of superoxide ions via the mitochondrial electron-transport chain, which results into the production of reactive oxidant species (ROS) [2 ,6]. This overall enhancement in ROS levels increases the oxidative stress which precipitates complications associated with uncontrolled glycemia [4, 6]. Recent reports indicate that ROS play a significant role the pathophysiology of DN [7]. Conversely, anti-oxidant supplementation has shown to ameliorate the symptoms associated with DN [8, 9, 5].

Also, neuro-protective agents such as MCA have shown to prevent peripheral nerve degeneration in pre-clinical and clinical settings [10, 11, 12, 13]. Previous study from our laboratory has shown that VE and MCA treatment ameliorated surgery induced neuropathy in rats [13, 14]. Additionally, early co-administration of VEM resulted into quick recovery in nerve conduction velocities of rats subjected to sciatic nerve crush injury [15].

Thus, early commencement of anti-oxidant supplementation along with a powerful neuro-protective agent could effectively prolong, if not prevent the progression of DN [5]. With this hypothesis, we aimed to determine the effect of early co-administration VEM on DN in laboratory rats. Previous pre-clinical studies have shown that clinical symptoms of DN such as TH and allodynia can be replicated in laboratory animals [16].

These symptoms have been measured using paradigms such as hot plate test and hot water tail immersion assays in rats and mice [17, 18]. Also, measurements of the nerve conduction velocity have been applied to determine axonal degeneration and nerve re-generation in diabetic rats [19].

Thus, for this current study, TH was assessed behaviourally using hot plate test and hot tail flick assay and MNCV was measured to determine nerve degeneration in alloxan-induced diabetic rats.

MATERIAL AND METHODS:

Animals: Adult male Wistar rats (250-300 g, National Toxicology Centre, Pune, India) were housed at the animal housing facility of Poona College of Pharmacy. The rat colony was maintained in a temperature (24±2°C) and humidity (relative humidity of 30-70%) controlled room. The lights for this room were maintained on 12:12 h cycle with lights ON from 0700 h. The rats were housed 3 per polycarbonate cage. All animals had free access to water throughout the experimental span.

All rats received standard animal pellets *ad libitum,* except prior to diabetes induction where all animals were fasted 48 h prior to vehicle/alloxan treatment. In order to minimize the handling stress, all rats were handled by the experimenter for 5 days prior to the commencement of experiments. Induction of diabetes, SGL estimation, behavioral testing for TH and MNCV measurements were performed between 0900 and 1700 h. The procedures used to determine pain parameters were in accordance to the guidelines mentioned by Zimmermann [20]. All experimental procedures used in this study were reviewed and approved by The Institutional Animal Ethics Committee of Poona College of Pharmacy, Pune, India.

Drugs and chemicals: Alloxan monohydrate (Loba Cheme, Munbai, India), MCA and pentobarbital sodium (gifted by Emcure Pharmaceuticals, Pune, India) were dissolved in physiological saline. VE (Hi Media, Mumbai, India) was suspended in 1% Tween 80 (Loba Cheme, Mumbai, India). Intraperitoneal (ip) injections were administered in a volume of 1 ml/kg. All drug weights refer to the salt.

Induction of diabetes: The SGL and behavioral parameters for TH (hot tail flick assay and hot plate test) were assessed in all animals, 3 days prior to the induction of diabetes. Diabetes was induced by methods described by Morani and Bodhankar [17, 18]. On the day of alloxan treatment, animals were divided into two groups, Sham treated group, which received only vehicle (0.9% saline, SC, n=6) and the other group of rats received alloxan injection (120 mg/kg, ip, n=16). All animals were fasted for 48 h prior to vehicle/ alloxan treatment.

The induction of diabetes was determined by measuring SGL 3 days post vehicle/alloxan treatment and based on the levels of SGL (>250 mg/dl taken as diabetic rat), animals were assigned into three separate groups - sham, diabetic untreated (DU) and diabetic treated (DVEM). The sham and DU group of rats received vehicle (used to suspend/dissolve VE and MCA) treatment, whereas the DVEM treated group received VEM [VE (50 mg/kg, ip) and MCA (500 µg/kg, ip)] treatment. All group of rats received corresponding vehicle / drug treatments once daily for 45 days following induction of diabetes, with treatments commencing from day 3 onwards.

Collection of blood and determination of serum glucose: SGL was estimated by using methods described by Badole and colleagues [21]. Animals were anaesthetized using ether (Loba Chem, Mumbai) anesthesia and blood samples were collected by puncturing the rat orbital sinus using heparinized capillary glass tubes (Loba Chem, Mumbai). The blood samples collected were analyzed for SGL by using glucose oxidase peroxidase kits (Accurex, Mumbai). The SGL was measured three days prior alloxan treatment, 30 and 45 days post alloxan treatment for all rats.

Hot water tail immersion assay: Methods described by Morani and Bodhankar [17, 18] were followed. This test was performed 3 days prior to alloxan treatment and 15, 45 days post alloxan treatment. Tail of rat was immersed in a warm water bath maintained at 50±0.1°C. The latency of tail flicking or withdrawal or signs of struggle were observed and were considered as end-point. To avoid tissue damage, a cut-off time of 18 sec was set for the test. The procedure was repeated thrice at an interval of 15 min and each time was carried out by a different blinded observer. A mean of these readings was taken as final response.

Hot Plate Test: This test was performed 3 days prior to alloxan treatment and 15, 45 days post alloxan treatment. TH was assessed using Ugo Basile Hot Plate Analgesiometer (Versace, Italy). Previous methods described by Morani and Bodhankar [15] were followed. The hot plate was maintained at 55±0.1°C and the paw withdrawal latency (PWL) was measured. A cut off time of 24 s was set to avoid tissue damage. The test was repeated thrice at an interval of 15 min and each time was carried out by a different blinded observer. A mean of these readings was taken as final response.

Motor Nerve Conduction Velocity: Previous methods described by Morani *et al*., [15,23] were followed. This test was performed on day 45 after the assessment of TH and SGL. Rats were anesthetized using pentobarbital sodium (50 mg/kg, ip). Body temperature of the animal was maintained at 37°C under a heating lamp. The right sciatic and tibial nerves were stimulated at sciatic and tibial notch respectively by a 0.1 ms square wave pulse delivered through a pair of monopolar needle electrode (1.0-1.5 mA, 2.0 mV/D). Responses were recorded from the indigital plantar muscles using Students Biopac data acquisition system (Santa Barbara, CA, USA). MNCV was determined by measuring the distance between sciatic and tibial nerve stimulation. The data was immediately recorded and printed and all calculations were verified by a blinded investigator.

Statistical analysis: Data analysis was performed using Prism Graphpad software (version 5.0). To analyze data for SGL, tail immersion assay, hotplate test and MNCV, separate one-way ANOVA's followed by Tukey post-hoc tests were applied. Differences between means were considered statistically significant for $p < 0.05$.

RESULTS AND DISCUSSION: Body weights for all animals were measured throughout the experimental span. Our observations showed that reduction in body weights were noted for the DU and DVEM group of rats, whereas, no significant reduction in body weights were noted for sham group of animals (results not shown).

Table 1 indicates the levels of SGL for the experimental animals. No significant difference in the SGL was noted for all treatment groups prior to alloxan treatment [P > 0.05, F (2,19) = 0.24]. However, on day 3, a significant increase in the SGL was noted for DU and DVEM treated group compared to sham treated group of animals (P < 0.05) [P < 0.0001, F (2,19) = 32.74]. Similar increases in SGL were observed for DU and DVEM group of rats compared to sham treated group on day 30 [P < 0.0001, F (2,18) = 48.43] and day 45 [P < 0.0001, F (2,18) = 88.58]. These results indicate an enhanced SGL for rats which received alloxan treatment compared to sham group of animals throughout the experimental span, thus indicating the induction of diabetes in rats which received alloxan.

TABLE 1: SERUM GLUCOSE LEVELS DURING EXPERIMENTAL SPAN.

Serum glucose levels (mg/dl).				
	Pre-alloxan treatment	**Day 3**	**Day 30**	**Day 45**
Sham (n=6)	91.56±11.08	103.12±9.22	101.6±23.87	95.32±20.21
DU (n=8)	105.91±19.09	298.2±27.3*	370.12±29.11*	394.21±18.32
DVEM (n=8)	100.3±8.76	318.22±12.98#	409.32±16.42#	399.24±15.39#

Sham: non-diabetic rats treated with vehicle; DU: diabetic rats treated with vehicle; DVEM: diabetic rats treated with VEM; *p<0.001, data compared for DU treated group vs. sham treated group on the corresponding days; #p<0.001, data compared for DVEM treated group vs. sham treated group on the corresponding days. One way ANOVA followed by Tukey post hoc test. Numbers in parenthesis indicate sample size.

Figure 1 indicates the measurement of TH using hot water tail immersion assay in rats. No significant difference in the tail withdrawal latency time was observed for all group of rats prior to diabetes induction [P > 0.05, F (2,19) = 0.05] and 15 days post alloxan treatment [P > 0.05, F (2,19) = 0.39]. A significant reduction in tail withdrawal latency was noted for DU treated rats compared to sham treated rats 45 days post diabetes induction [P < 0.05, F (2,18) = 6.6]. This, however, was not noted for DVEM treated group of animals (P > 0.05). These results indicate improvement in tail withdrawal latency for diabetic animals which received VEM treatment vs. DU group of rats (P < 0.05).

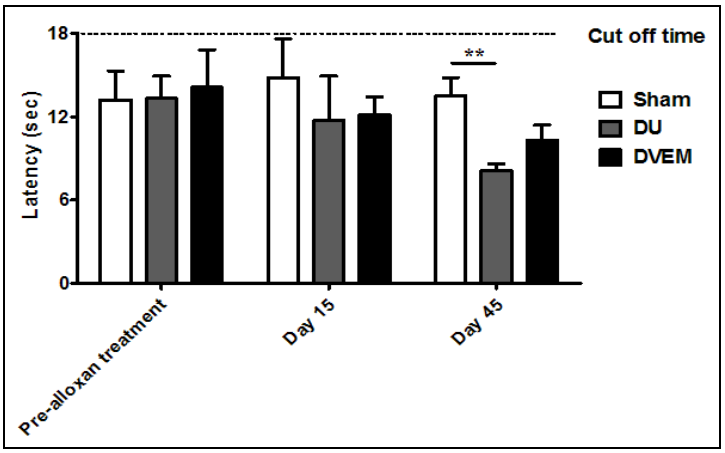

FIG. 1: EFFECT OF VEM ON HOT TAIL IMMERSION TEST

Bars indicate average (±SEM) time (in seconds) taken to withdraw tail immersed in hot water for sham, DU and DVEM treated group of rats. **p<0.01, data compared for DU treated vs. sham treated group of rats measured on day 45. One way ANOVA followed by Tukey post-hoc tests. n = 6-8 for each treatment groups.

Figure 2 shows the behavioral measurement of TH using hot plate test in rats. No significant difference in paw withdrawal latency was observed for all group of rats prior to diabetes induction [day 0; P > 0.05, F (2,19) = 0.3] and 15 days post alloxan treatment [P > 0.05, F (2,19) = 0.18]. A significant reduction in paw withdrawal latency was noted for DU treated rats compared to sham treated rats 45 days post diabetes induction [P < 0.05, F (2,18) = 5.2]. This, however, was not noted for DVEM treated group of animals (P > 0.05), thus indicating an improvement in paw withdrawal latency for diabetic animals which received VEM treatment vs. untreated diabetic rats (P < 0.05).

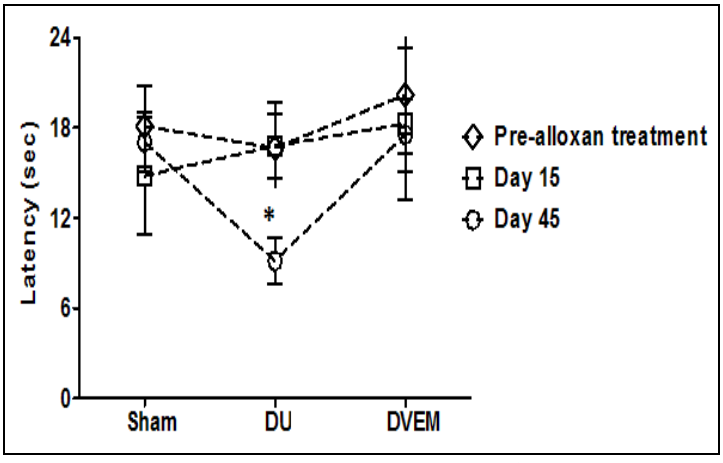

FIG. 2: EFFECT OF VEM TREATMENT ON HOT PLATE TEST

Symbols indicate average (±SEM) of paw withdrawal latency measured in seconds for sham, DU and DVEM treated group of rats. *p<0.05, data compared for DU treated vs. sham treated group of rats measured on day 45. One way ANOVA followed by Tukey post-hoc tests. n = 6-8 for each treatment groups

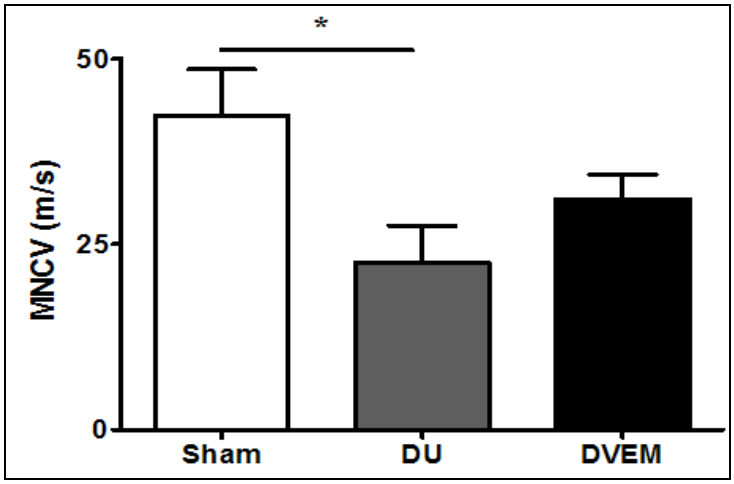

FIG. 3: EFFECT OF VEM TREATMENT ON MOTOR NERVE CONDUCTION VELOCITY

Bars indicate average (±SEM) of motor nerve conduction velocity (m/s) for sham, DU and DVEM treated group of rats. *p<0.05, data compared for DU treated vs. sham treated group of rats. One way ANOVA followed by Tukey post-hoc tests. n = 6-8 for each treatment groups.

Figure 3 indicates the MNCV measurement on day 45 for experimental animals. A significant reduction in MNCV for DU group of animals was observed compared to sham treated rats [P < 0.05, F (2,18) = 3.94]. However, post hoc tests showed no significant difference in MNCV for sham and VEM treated animals (P > 0.05).

Results from our present study demonstrates that early co-administration of VE and MCA prevents the development of neuropathic pain symptoms such as TH **(Figure 1, 2)** and improves MNCV **(Figure 3)** in alloxan induced diabetic rats. These results suggest the preventive role of VEM treatment in the development of DN symptoms. This effect might be attributed to the anti-oxidant effects of VE and the neuro-protective effects produced of MCA. As mentioned previously, enhanced levels of oxidative stress can lead to the C-fiber damage and further lead to the axonal de-generation[4, 6]. This had been observed symptomatically by the reduction in the latency for hot plate test and hot water tail immersion assay in diabetic rats (present study) [17, 18].

Our results from previous study showed that VEM treatment for 15 days attenuated TH and improved MNCV in surgery induced neuropathy in rats [22, 15]. These results prompted us to study the effect of early co-administered VEM on the progression of neuropathic symptoms in diabetic rats.

The results from our present study confirm the ameliorative effects produced by VEM co-administration on the development of neuropathic complication in diabetic rats. The mechanism by which VME prevents neuropathic pain behavior needs further explanation. Therefore, future work in this direction is warranted.

One of the limitation of this current study was that both DU and DVEM group of animals had uncontrolled glycemia **(Table 1)**. This limitation in the experimental design raises questions on what would be the effect of VEM treatment along with controlled glycemia on the progression of DN. From our previously published reports, we observed a marked improvement in pain thresholds in rats with controlled glycemia and anti-oxidant supplementation [17, 18].

With promising results from this present study, it would be interesting to determine the combined effect of VEM supplementation and glycemic control on progression of DN.

CONCLUSIONS: Given that oxidative stress plays a prominent role in the development of diabetic complications and peripheral nerve degeneration, our present results indicate ameliorative effects of VME treatment on the development of neuropathic complications in diabetic rats. These results have potential to unearth novel strategies in developing preventive therapeutics for diabetic complications. However, further research is warranted to establish this hypothesis.

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