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Original Research Article

Valproic acid and ivermectin increase GABA levels to improve the viability of skin flaps in rats

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ABSTRACT

Background: Gamma-aminobutyric acid (GABA) is a nonproteinogenic amino acid known as the main inhibitory neurotransmitter in the central nervous system. Ivermectin (IVM) and valproic acid (VA), both increase GABA levels. The purpose of this study was to determine the effect of VA and IVM on the viability of extended random-pattern skin flaps in Wistar rats and their GABA-dependent mechanisms.
Methods: This experimental study used 32 Wistar rats that underwent surgery to have caudally based extended random-pattern skin flaps divided into four distinct groups. In the first group, 0.05 mg/kg IVM was administered via intraperitoneal (i.p.) injection 30 minutes (min) prior to elevating the flap. The second group was administered 100 mg/kg VA by i.p. injection 60 min prior to elevating the flap. The third group was administered VA 100 mg/kg followed by IVM 0.05 mg/kg injected (i.p.) 60 min and 30 min prior to flap elevation, respectively. The fourth group was used as a control. After 7 days, the percentage of flap viability was measured, and tissue sampling was performed to examine GABA levels.
Results: It was found that the highest viability rate was in the group administered VA combined with IVM (93.98%) compared to all other groups ($p<0.001$). The highest GABA levels in the tissue were observed in the group administered VA combined with IVM (284.91 nmol/l) compared to all other groups ($p<0.001$).
Conclusions: IVM in combination with VA improves the viability of extended random-pattern skin flaps by increasing GABA levels.

Keywords: Extended random skin flap, Flap necrosis, GABA, Ivermectin, Valproic acid

INTRODUCTION

In plastic and reconstructive surgery, random-pattern skin flaps are frequently used, and their viability depends on small musculocutaneous or subcutaneous arteries for perfusion. Skin flap necrosis is a serious side effect of flap surgery that can lead to several interventions and the need for additional surgical procedures. The distal flap necrosis is a result of severe ischaemia due to venous and arterial flow obstruction.

Skin flaps with random patterns are frequently used to improve and restore functionality to skin defects, deep wounds, congenital skin blemishes, and cancer-related deformities. When the flap's length-to-width ratio is more than 2:1, inadequate blood flow may lead to necrosis. In random-pattern skin flaps, flap necrosis mostly occurs at the distal end of the flap. Insufficient venous outflow, insufficient arterial blood supply, or a combination of the two can lead to distal flap necrosis.[1] Researchers around the world invest their efforts in developing new strategies that improve tissue viability after ischaemic events.

Tissue conditioning can be used in order to prevent or reduce ischaemia, adapt the damaged tissue to the stress that follows during and after ischaemia, and prevent or minimise ischaemia–reperfusion (I/R) injury.[2]

Partial or total necrosis of the skin flap is primarily caused by inadequate blood supply to the flap. The ischaemic area of flaps is generally more distal to the region of vascular supply. Reactive oxygen species (ROS) can be produced by ischemia, which happens quickly. Following ischaemia, there is a period of time known as reperfusion, which can cause inflammation and alterations in cell metabolism while restoring blood flow and oxygen intake to the ischemic tissues. Free radicals are responsible for these alterations, as they can lead to tissue necrosis and alter the structure and function of cells.[3]

For I/R there are, three different therapeutic approaches that can be implemented. These approaches can be used either before, during, or after ischaemia. Ischemic preconditioning has drawn more attention recently as an ideal preventative measure against I/R injury with numerous benefits, including high efficacy, safety, noninvasiveness, ease of use, and affordability.[4] Furthermore, a variety of therapeutic agents have been created to treat I/R injuries, such as synthetic medications like cyclosporine, natural chemicals like cannabinoids, endogenous gases (e.g., endogenous gases, carbon monoxide, and hydrogen sulphide), and stem cell therapies. Endogenous chemicals or signals may be a better target due to the negative effects of chemical drug administration and the expensive expense of stem cell therapy. GABA is widely distributed throughout the neural system and is thought to be connected to I/R damage.[4]

The central nervous system's main inhibitory neurotransmitter is GABA, a nonproteinogenic amino acid.[4] Its physiological function involves modulating synaptic transmission and safeguarding several organs against ischemia/reperfusion injury, including the kidney, gut, cerebellum, and spine. Furthermore, it has been demonstrated that GABA receptor stimulation suppresses inflammation, which is one of the primary mechanisms underlying I/R damage. GABA interacts with mechanoreceptors in skin tissue. Skin tissue also has GABA receptors. Furthermore, it has been shown that activating GABA types A and B in the skin may have antipruritic effects that are mediated by immune cell regulation.[5] Antigen-presenting cells and other immune cells exhibit GABA receptors, particularly type A receptors, which are clearly suppressive of the immune system and reduce inflammation.[6]

IVM was first employed as an antiparasitic agent. A variety of mechanisms, such as interactions with the GABA type A receptor and activation of ligand-gated chloride channels, particularly glutamate-gated chloride channels, have been hypothesised to mediate these effects. Furthermore, it has been shown that GABA receptor stimulation suppresses inflammation, which is one of the primary mechanisms underlying I/R damage.[5]

VA is an anticonvulsant drug that significantly increases GABA receptor expression. Antigen-presenting cells have GABA receptors, and activation of these receptors helps to reduce inflammation.[6]

The main purpose of this study was to evaluate the effect of ivermectin combined with VA on the viability in extended random skin flaps of Wistar rats and their GABA-dependent mechanisms.

METHODS

Animal management

This was an experimental study, thirty-two Wistar rats weighing between 250 and 300 grams were employed. Animals were obtained from the animal house of the pharmacology department, Udayana University of Medical Sciences (Indonesia) in December 2022 to January 2023. The animals were housed in plastic cages at room temperature (25-30°C) in the animal house both before and after the elevation flap. They had free access to clean water and enough food. To minimize stress, every surgery and injection was given to an individual animal apart from the others. The ethics in medical research committee granted approval for this study (number 2740/UN14.2.2.VII.14/LT/2022).

Preparation of Ivermectin and Valproic Acid

Before lifting the flap and taking tissue samples, each rat received an intramuscular (i.m.) injection of a mixture of xylazine 2% (10 mg/kg) and ketamine 10% (100 mg/kg) (Saputro, Budi, and Fiona, 2022). IVM was dissolved to a final dosage of 0.05 mg/kg in sterile water. VA was dissolved to a final dosage of 100 mg/kg in sterile water. According to the intended experiment, solutions were intraperitoneally injected before the flap was raised.

Random-pattern skin flap surgery

In this work, an extended random-pattern skin flap surgery was carried out. Following the administration of general anesthesia and the shaving of the rats' dorsal hair, each rat's dorsum was cleaned, and a 1.5 × 6 cm rectangular section was marked with a marker. The caudal edge of the flap was located anatomically, using the hip joint as a reference. The flap was lifted, its edges cut with a surgical blade, and its caudal side was left attached to its original location. After raising the flap tissue, the major caudal artery supplying the skin flap tissue was meticulously located. After that, the major artery was severed to create ischemia and a skin flap with an erratic pattern. In order to assure ischemia, the flap was then kept open at the location and wrapped with sterile gauze saturated in regular saline. The flap was then

continuously stitched with 4/0 nylon to return it to its original location.

Study design

To evaluate the possible involvement of the GABAergic system in skin flap survival, in the first group, 0.05 mg/kg IVM was i.p. injected 30 minutes prior to elevating the flap, as previously mentioned. IVM was used as a GABA agonist. In the second group, 100 mg/kg BW VA was i.p. injected 60 minutes prior to elevating the flap. VA was used as an agent that vastly upregulates the expression of GABA receptors. In the third group, VPA was co-administered with IVM to examine the possible synergistic effect. The fourth group, as a control, received only normal saline 30 minutes prior to elevating the flap.

Data collection and tissue sampling

Rats were typically given the same treatment for ketamine and xylazine anesthesia on the seventh day following the skin flap procedure. All study groups used the same camera and set of environmental parameters to take digital photos of each rat's flap region on its dorsum. Necrotic skin is defined as having dark-coloured, thickened, and rigid tissue. Healthy skin is soft and flexible pink skin. In the end, ImageJ software version 1.52 was used to compute each flap's necrotic area as follows: flap viability rate area (%) = (area of viable skin flap divided by area of whole flap) ×100.[5] In order to sample tissue, full-thickness skin was removed from the 1×1 cm border between the necrotic and grossly healthy areas. Then, this tissue segment was rinsed in cold phosphate-buffered saline (PBS) with a pH of 7.4 and stored at 4°C for evaluation by enzyme-linked immunosorbent assay (ELISA).

Measurement of GABA levels

Quantitative assessment of GABA in tissue skin flaps was performed using a rat-specific ELISA kit between the treatment and control groups. The specimens were finely chopped and homogenized. The homogenate was then centrifuged for 15 minutes at 12000 RPM at 4°C to obtain the supernatant. Following an adjustment for protein content, the final concentration was reported as nmol/l.

Statistical analysis

The data on the percentage flap viability rate (FVR) of the skin flap are reported as the mean±SD in each group. The GABA levels in the tissue skin flaps and the ELISA test findings are presented as the mean±SD for each group. Homogeneity of variance tests were used to verify that the data had a normal distribution. The percentage flap viability rate of the skin flap and the ELISA data were analysed using one-way ANOVA and the LSD post hoc test. The Statistical Package for the Social Sciences (SPSS) was used to conduct and present the analyses. A significant p value was defined as one that was less than 0.05.

RESULTS

The effect of ivermectin on skin flap viability: evaluating the role of GABA

After day 7, the mean percentage of the viable area was 68.44±5.93% in the control group. In the group that received IVM alone, IVM 0.05 mg/kg was i.p. administered 30 minutes prior to elevating the flap. After 7 days, the mean percentage of the viable area was 78.20±6.59%, which was significantly higher than that of the control group ($p<0.01$).

The effect of valproic acid on the viability of skin flaps: evaluating the role of GABA

In the VA only group, 100 mg/kg VA was i.p. injected 60 min prior to elevating the flap. The mean percentage of the viable area was 81.59±3.98%, which suggested that VA showed a protective effect against necrosis as compared with that of the control treatment ($p<0.001$). The mean percentage was also significantly higher than that in the IVM 0.05 mg/kg group (mean: 78.20±6.59%, $p>0.05$), which was not significantly different from the IVM group.

The coadministration of valproic acid and ivermectin on skin flap viability: evaluating the role of GABA

In the combination treatment group, VA (100 mg/kg) followed by IVM (0.05 mg/kg) were i.p. injected 60 minutes and 30 minutes prior to flap elevation, respectively. The mean percentage of the viable area in this group was 93.98±2.48%, which, compared to the control treatment, demonstrated a protective effect against necrosis ($p<0.001$). Additionally, this group's mean percentage was statistically higher than the 0.05 mg/kg IVM alone group. (mean: 78.20±6.59%, $p<0.001$) and the 100 mg/kg VA only group (mean: 81.59±3.98%, $p<0.001$) (Figures 1 and 2).

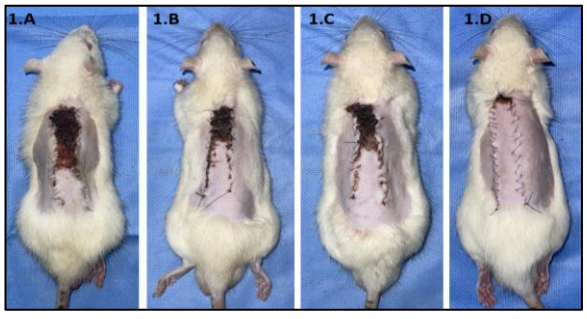

Figure 1: The appearance skin flap 7 days after treatment with different does with different groups; (A) control group (B) ivermevtin group, (C) valproic acid group (D) valproic acid and ivermectin group.

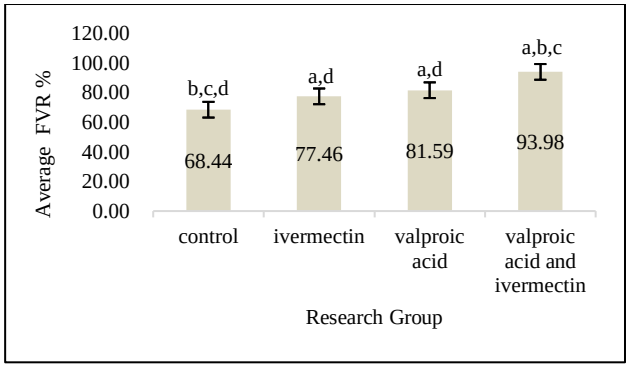

Figure 2: Post hoc test with different average levels of FVR; (a) significantly different from control, (b) significantly different from ivermectin, (c) significantly different from valproic acid, (d) significantly different from valproic acid and ivermectin (p<0.001).

GABA ELISA results

The mean GABA levels in the control group's skin flap tissue were 162.89±2.74 nmol/l. After IVM (0.05 mg/kg) was administered, the skin flap tissues had a noticeably greater amount of GABA; the mean level of GABA was 197.39±1.61 nmol/l (p<0.001 compared to the control group). The mean GABA levels in the skin flap tissue of the VPA (100 mg/kg) group (228.17±2.18 nmol/l) were significantly higher than those compared to the control group (p<0.001). After the coadministration of VA (100 mg/kg) and IVM (0.05 mg/kg), the mean GABA levels in the skin flap tissue were 284.90±8.49 nmol/l (p<0.001), which were also significantly higher than those compared to the other groups (Figure 3).

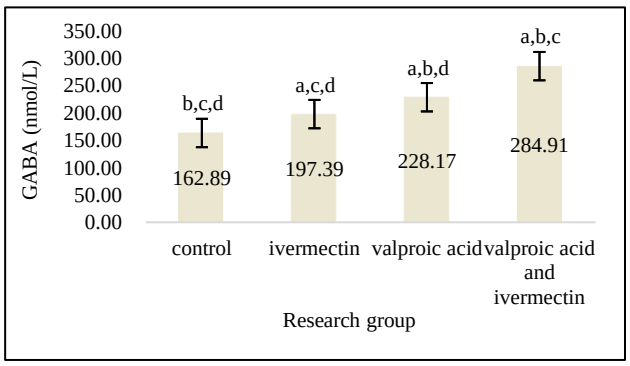

Figure 3: Post hoc test with different mean GABA levels; (a) significantly different from control, (b) significantly different from ivermectin, (c) significantly different from valproic acid, (d) significantly different from valproic acid and ivermectin (p<0.001).

DISCUSSION

The study's primary findings, which were highlighted, showed that VPA and IVM enhanced the survivability of skin flaps, and that this enhancement was probably mediated by elevated GABA levels. The most improved skin flap viability was observed in the group that was coadministered VA (100 mg/kg) and IVM (0.05 mg/kg) in our study. Additionally, there was a greater degree of expression of GABA in the group that was administered both VA (100 mg/kg) and IVM (0.05 mg/kg) compared to that in all other groups after random-pattern skin flap surgery.

IVM, a naturally produced avermectin B1 is believed to operate via a variety of ligand-gated channels, such as GABA receptors.[7,8] GABA has three distinct types of receptors: metabotropic GABAB receptors, ionotropic GABAA, GABAC receptors, and both types of receptors. GABA is mostly synthesised from glutamate.[4] It has been demonstrated that GABA might provide protection in cerebral tissue from ischemia/reperfusion (I/R) damage. This protective effect could be brought about by the increased GABA pathway's possible inhibition of glutamate release.[5] Additionally, some research has demonstrated that in the adult central nervous system, GABA predominantly inhibits neuronal excitability of the postsynaptic membrane and that GABA agonists protect against cerebral ischaemia.[4]

IVM blocks the reception of both excitatory and inhibitory signals by inducing GABA's presynaptic release, and opening the muscle cell's postsynaptic membrane's chloride-ion channels. Which, in the case of an excitatory neurone's signal, is believed to be triggered by the presynaptic release of glutamic acid.[8]

In line with earlier research on the function of GABA in the regulation of inflammatory reactions, our data also revealed that GABA levels in the tissue of the skin were higher following the administration of IVM, suggesting a reduction of the inflammatory process. The expression of GABA receptors in immune cells has the ability to inhibit the release of cytokines.[9]

Prior research has also demonstrated that the administration of agents from the IVM family results in an increased expression level of GABA receptors. After being exposed to IVM, pigeons demonstrated increased expression levels of GABAA and GABAB receptors, according to a study by Chen et al. Furthermore, GABAA receptor subunit expression was elevated.[10] The target tissue and the duration after I/R injury have a major influence on GABA receptor expression.[5] GABAA a1 subunit and GABAB R1 subunit 7 days after surgery were shown to be expressed at higher levels in the skin; however, additional research is required to determine these levels prior to and following this time point.[5]

In a recent study, Ala et al. suggested that the GABAergic system-mediated protective impact of sodium valproate on skin flap survival.[6] The function of GABA in the viability of skin flaps, because these are the most significant known targets of sodium valproate. The group receiving VA treatment at a dose of 100 mg/kg

showed the highest survivability of skin flaps. When VA (100 mg/kg) and IVM (0.05 mg/kg) were administered together, the results were completely different than when VA, IVM, and control were administered separately. This suggests that a GABA-dependent pathway may be involved in reducing skin damage brought on by ischaemia.[6]

VA increases GABA levels by inhibiting GABA degradation, increasing GABA synthesis, and decreasing GABA reuptake. In vitro studies show that VA increases glutamic acid decarboxylase (GAD) activity, which increases GABA synthesis and inhibits GABA-T (GABA Transaminase), an enzyme that degrades GABA.[11] Pharmacokinetic studies suggest that VA is quickly absorbed when taken orally, peaking in the blood in one to four hours.[12] This rapid absorption was the reason why, in this study, the authors administered an injection of VA 1 hour prior to the elevation of the flap.

In this study, skin flap viability and GABA levels were statistically correlated with each other with a very strong correlation ($p<0.001$, r: 0.891, correlated 79.39%). There is a correlation between IVM and VA, where the mechanism of action is to increase the GABA levels in the flap tissue. However, there are differences in the mechanism of action, namely, IVM stimulates GABA-mediated chloride ion conduction, acts as a GABA agonist, stimulates presynaptic GABA release, and potentiates GABA binding to receptors. The mechanism of IVM is the activation of glutamate-gated chloride channels, causing the entry of chloride into the cell, which increases postsynaptic membrane polarization and makes excitation and action potentials difficult. The mechanism of VA increases GABA levels by inhibiting GABA degradation, increasing GABA synthesis, and decreasing GABA reuptake. In vitro studies show that VA increases glutamic acid decarboxylase (GAD) activity, which increases GABA synthesis and inhibits GABA-T (GABA Transaminase), an enzyme that degrades GABA.[11]

Limitations of the study is, it did not examine the microstructure of the skin, which showed that the epithelial layer had a better structure after being treated with valproic acid and ivermectin to see the number of inflammatory cells, necrotic cells, regenerative cells, and the distribution of blood vessels. For the next study, we suggest follow-up with histopathological and immunohistochemical tests.

CONCLUSION

Our findings indicate that coadministration of VA and IVM could improve the skin flap survival of extended random-pattern skin flaps in Wistar rats, and this improvement is mediated by GABA transmission and statistically significant compared to that in all other groups after random-pattern skin flap surgery. This study finding may support the repurposing of this old drug.

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Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee, Faculty of Medicine, University of Udayana of Medical Sciences (No. 2740/UN14.2.2.VII.14/LT/2022)

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