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ORIGINAL ARTICLE

PROFOUND SWIM STRESS-INDUCED ANALGESIA WITH KETAMINE

Asma Hayati Ahmad, Zalina Ismail, Myo Than***, Azhar Ahmad***

**Department of Physiology, *Department of Chemical Pathology,
**Deputy Dean's Office, School of Health Sciences, School of Medical Sciences,
Universiti Sains Malaysia, Health Campus
16150 Kubang Kerian, Kelantan, Malaysia**

****Department of Anatomy, Perak College of Medicine, 30450 Ipoh, Perak, Malaysia**

The potential of ketamine, an N-methyl D-aspartate (NMDA) receptor antagonist, in preventing central sensitization has led to numerous studies. Ketamine is increasingly used in the clinical setting to provide analgesia and prevent the development of central sensitization at subanaesthetic doses. However, few studies have looked into the potential of ketamine in combination with stress-induced analgesia. This study looks at the effects of swim stress, which is mediated by opioid receptor, on ketamine analgesia using formalin test. Morphine is used as the standard analgesic for comparison. Adult male Sprague-Dawley rats were assigned to 6 groups: 3 groups (stressed groups) were given saline 1ml/kg intraperitoneally (ip), morphine 10mg/kg ip or ketamine 5mg/kg ip and subjected to swim stress; 3 more groups (non-stressed groups) were given the same drugs without swim stress. Formalin test, which involved formalin injection as the pain stimulus and the pain score recorded over time, was performed on all rats ten minutes after cessation of swimming or 30 minutes after injection of drugs. Combination of swim stress and ketamine resulted in complete analgesia in the formalin test which was significantly different from ketamine alone ($p<0.05$) and saline with stress ($p<0.01$). There is no significant difference between ketamine stressed and morphine stressed. These results indicate that ketamine and swim stress act synergistically to produce profound analgesia in the formalin test. This suggests that in the clinical setting, under stressful situations such as operative stress, ketamine is capable of producing profound analgesia at a subanaesthetic dose.

Key words *: ketamine, morphine, formalin test, swim stress.*

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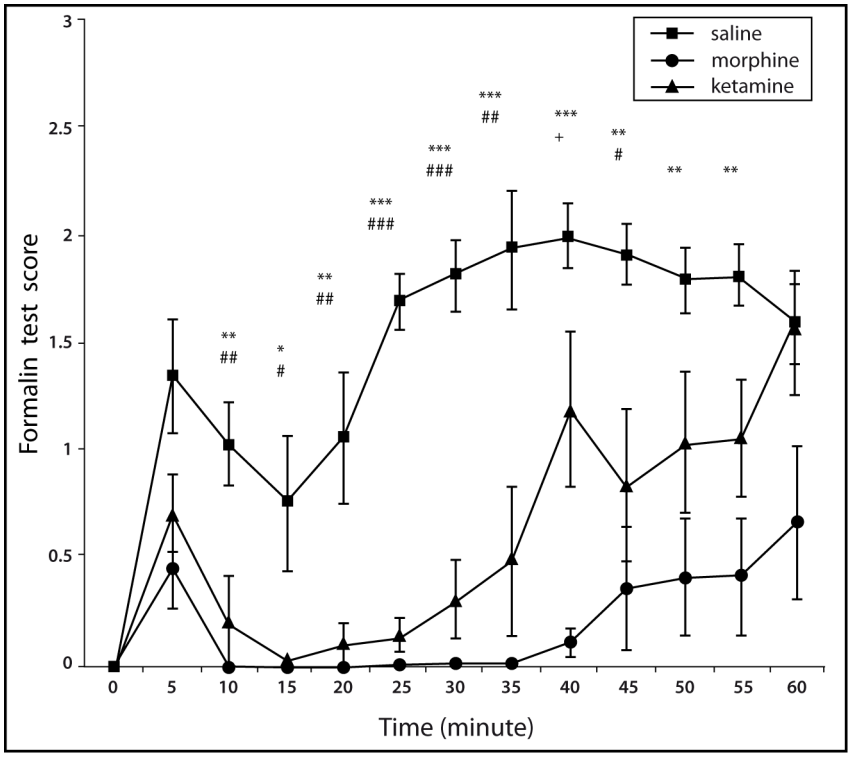
Introduction

Studies have identified the pathway of pain commencing from the site of stimulation to the spinal cord and subsequently the brain. One breakthrough finding is the study which showed that noxious stimulation in the periphery is able to cause the expression of a gene named c-*fos* in the corresponding spinal cord segment mediated via the N-Methyl-D-Aspartate (NMDA) receptor (1). The importance of c-*fos* is that it is implicated in the development of central sensitization (2), a phenomenon brought about by changes in the pain pathway due to neuroplasticity in the central nervous system (3). One drug that has the potential to reduce the development of central sensitization is ketamine, an NMDA receptor antagonist (4). Low dose ketamine is increasingly used to provide analgesia without producing side effects usually produced by its normal anaesthetic dose (5) and it is also widely used in preemptive analgesia (6).

The central nervous system possesses intrinsic

*Figure 1: Mean formalin test scores in non-stressed groups against time. n=8 for all groups. Values are means ± S.E.M. * p<0.05, ** p<0.01, *** p<0.001 for comparison between morphine and saline. # p<0.05, ## p<0.01, ### p<0.001 for comparison between ketamine and saline. + p<0.05 for comparison between morphine and ketamine.*



pain suppression systems (7) which are activated by noxious stimulation (8). It is also the basis for stress-induced analgesia (SIA) (9), whereby different forms of stress can produce potent analgesia (10). The factors involved in the induction of SIA include intensity of the stress stimulus, duration, and temporal aspects i.e. whether the stimulus is applied continuously or intermittently (11). SIA plays an important role in the survival of animals especially in fight-or-flight situations (9). This phenomenon is particularly difficult to study in humans (12) but its existence is confirmed by various studies (13, 14). Among the earliest reports of SIA in humans are observations done by Beecher, as reported by Koltyn (15), who found that soldiers severely wounded in battle reported little pain and required considerably less analgesic medication compared with civilians undergoing similar surgery.

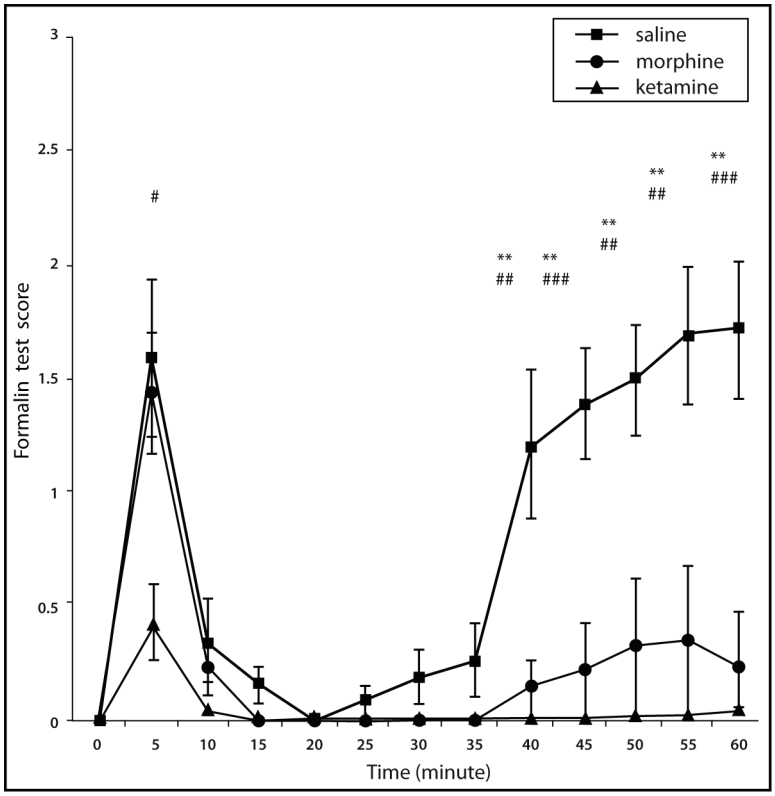
Assessment of analgesia in experimental animals employs the use of pain tests such as the tail flick test, the hot plate test or the formalin test. Formalin test is widely used to assess analgesia produced by various stressors, including swim stress (16). It has a peculiar two-phase response produced by different mechanisms which makes it an ideal instrument in pain research (17). Ultimately, there is involvement of the NMDA receptor (18) as a result of repetitive peripheral nociceptive impulses mediated through C fibres resulting in increased central excitability of dorsal horn neurons (19). With NMDA receptor involvement, the formalin test inevitably causes induction of c-*fos* mRNA and subsequently Fos protein expression which allows quantification of the pain response (20; 21).

In this study, experimental animals were subjected to swim stress to produce SIA, and the resultant analgesia is measured using formalin test as the pain test. Morphine, the gold standard for analgesics (22), and low dose ketamine were given prior to stress-induced analgesia. Both these drugs are widely used in clinical practice as analgesics and/or for the prevention of neuroplasticity and central sensitization (23, 4). The objective of this study is to assess the analgesia produced by a subanaesthetic dose of ketamine alone and in combination with swim stress in the rat formalin test.

Materials & Methods

Animals

Figure 2 : Mean formalin test scores in stressed groups against time. Values are means ± S.E.M. *p<*0.05,* **p<*0.01,* ***p<*0.001 for comparison between morphine and saline.* # p<*0.05,* ## p<*0.01,* ### p<*0.001 for comparison between ketamine and saline.*



Adult male Sprague-Dawley rats, weighing between 230-350g, were maintained in a 12-h light dark cycle and allowed free access to food and water. Rats obtained from the Animal House were housed in individual cages and allowed adaptation for at least four days in the Department of Physiology laboratory. Each animal was used only once. Experiments were performed between 0800 and 1600 in the same department's laboratory. This study was approved by the Animal Ethics Committee and Research Committee of Universiti Sains Malaysia.

Vehicle Used in Experiment

All drugs and saline controls were administered as pretreatment i.e. before the swim stress and formalin test procedures. Saline 0.9% (Sigma) was used as vehicle to dissolve the drugs. The drugs used were:

1) Ketamine (Gedeon Richter Ltd.) 5mg/kg, intraperitoneal
2) Morphine (Duopharma (M) S/B) 10mg/kg, intraperitoneal
3) Saline (Sigma) 0.9% as control

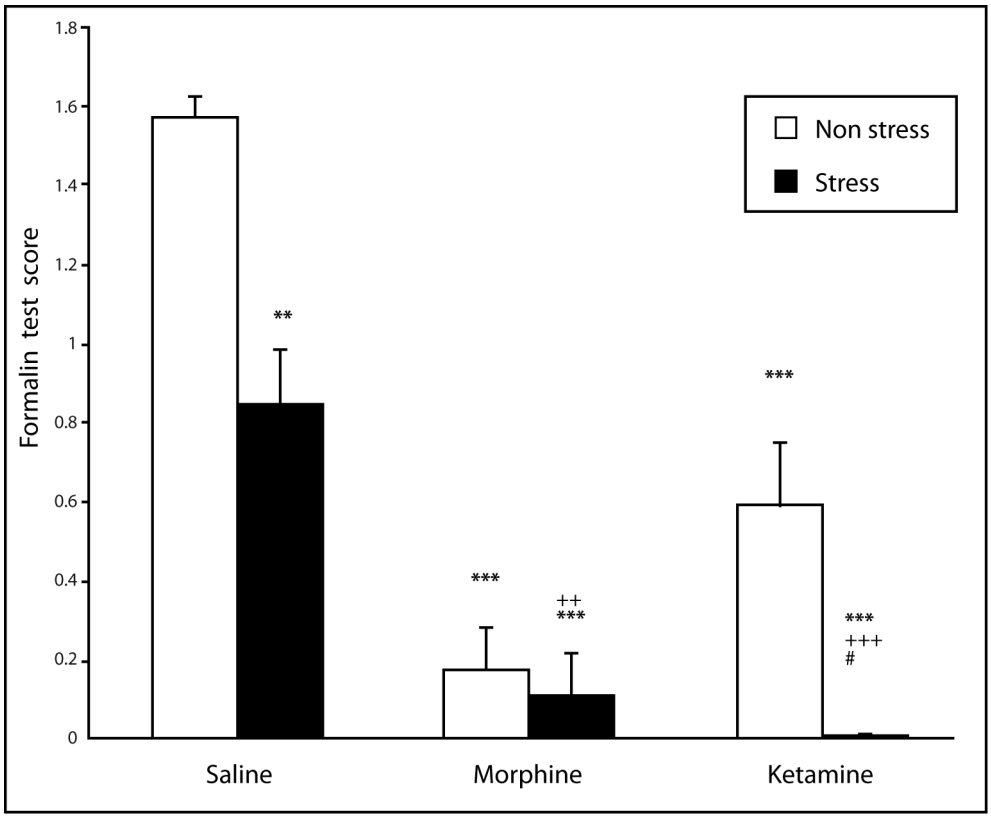
The dosage used for ketamine were a subanaesthetic dose (24, 25, 26) whereby the rats would experience loss of righting reflex for about five minutes only and would have recovered fully before undergoing swim stress. The dosage for morphine was one that gave analgesic in the rat formalin test (27, 28). Morphine was the gold standard against which the analgesic or antinociceptive activities of other compounds were compared (29).

Experimental Groups

Rats were allocated to one of six experimental groups with eight animals in each group. The experimental group A (non-stressed group) consisted of one group of rats pretreated with ketamine, second group of rats pretreated with morphine and the third group of rats pretreated with saline. Formalin test was carried out 30 minutes after pre treatment to allow time for the action of each drug to reach its peak (30-31, 28).

The experimental group B (stressed group) consisted of the first group of rats pretreated with ketamine, second group of group rats pretreated with

*Figure 3 : A comparison of mean formalin test scores during phase 1 of non-stressed and stressed groups. n=8 for all groups. Values are means ± S.E.M. * p<0.05 compared with saline stressed.*



morphine and third group of rats pretreated with saline

Animals in this group received similar pretreatment as Group A. Fifteen minutes after pretreatment (30, 31) they were subjected to three minutes (32, 33) of swim stress. Ten minutes after cessation of swimming, formalin test was performed on all the rats. Ten minutes is the time of peak antinociception following swim stress (32). The timing is set thus so as to equalize the time interval between drugs administration and pain stimulation for both the stressed and the non stressed groups.

Acute Swim Stress Procedure

A container measuring 92 cm x 46 cm x 46 cm high containing 20 cm of water (30; 32; 25) at 20°C (30, 33) was used for this purpose. Rats were placed in the water individually and left to swim for three minutes before being removed (32; 34).

Formalin Test

Formalin test was performed 10 minutes after cessation of acute swim-stress. Diluted (1%) formalin (35) was prepared freshly from 37% formaldehyde with 0.9% normal saline before use (36), 50 µl was injected subcutaneously into the plantar surface of the right hindpaw using a 27-gauge needle (28). The rat was then placed in a perspex testing chamber measuring 26cm x 20cm x 20cm. A mirror was placed below the floor of the chamber at 45° angle to allow an unobstructed view of the rat's paws (27, 37, 38). The amount of time spent in each of four behavioural categories, 0-3, was recorded with a videocam (39) starting from the time of injection until the end of one hour. The tape was later viewed by two observers blinded to the treatment of each rat and the formalin test score was tabulated every minute and averaged at 5-minute intervals (35). The quantification was based on the total time spent in 4 behavioural categories (27). The categories were:

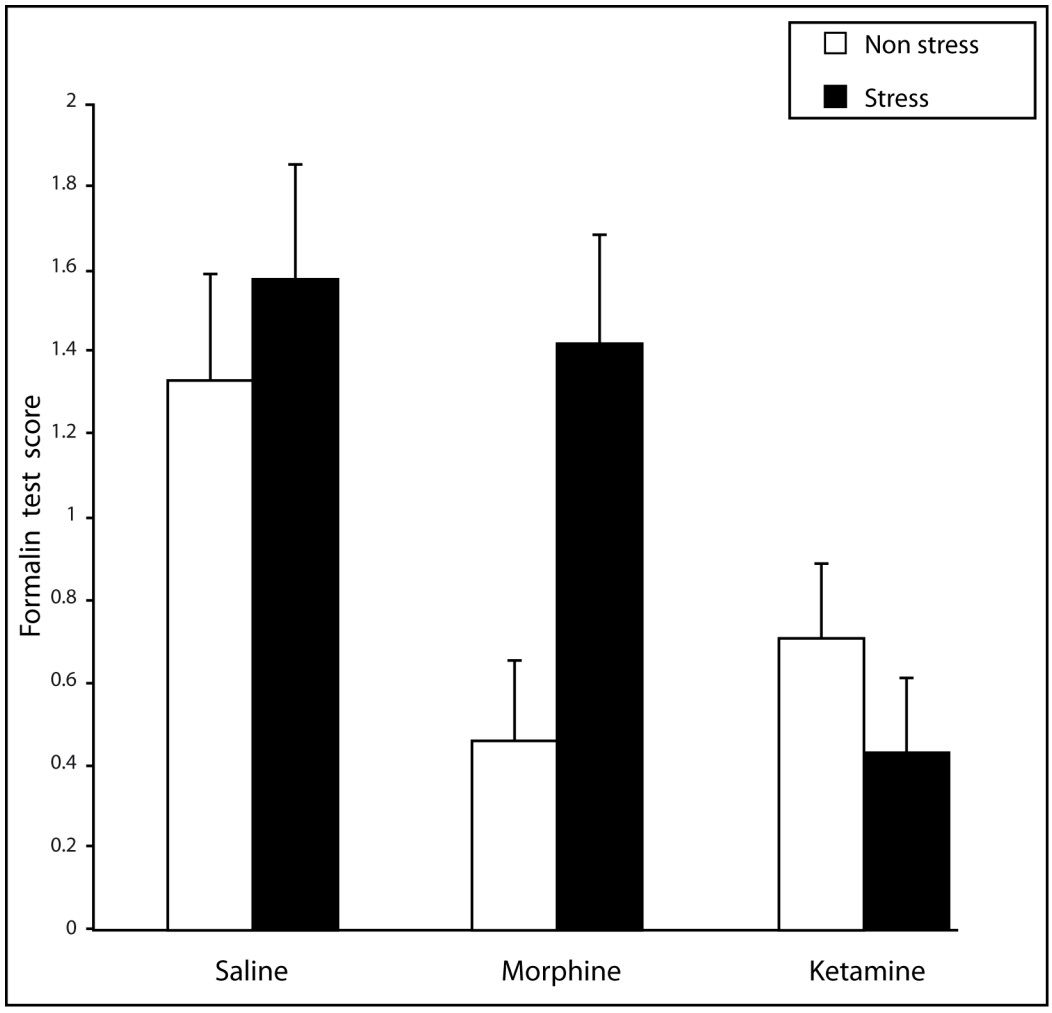
0 - the injected paw was not favoured (i.e. foot flat on the floor with toes splayed) indicating insignificant or no pain felt

1 - the injected paw had little or no weight on it with no toe splaying indicating mild pain felt

2 - the injected paw was elevated and the heel was not in contact with any surface indicating moderate pain

3 - the injected paw was licked, bitten or shaken indicating severe pain All rats were used only

*Figure 4 : A comparison of mean formalin test scores during phase 2 of non-stressed and stressed groups. n=8 for all groups. Values are means ± S.E.M. **p<0.01, *** p<0.001 compared with saline non-stressed ++ p<0.01, +++ p<0.001compared with saline stressed # p<0.05 compared with ketamine non-stressed*



once and sacrificed after experiment.

Statistical analysis

Pain behaviour scores by formalin test were analyzed using repeated measures analysis of variance (ANOVA) with *post hoc* Scheffé's test. One-way ANOVA was used to calculate significant differences at each time point, as well as effects of Phase 1 formalin test (mean score at 5 minutes) and Phase 2 (mean of scores from 10 to 60 minutes) (17). Significance was accepted at $p<0.05$.

Results

Effects of ketamine and morphine on rats' behaviour

Observation of the rats immediately after injection of drugs showed that rats receiving ketamine 5mg/kg demonstrated loss of righting reflex for about 2 to 3 minutes and recovered fully before being subjected to swim stress. This showed that the dosage used only had transient anaesthetic effect and did not affect performance during the swim stress procedure. Rats receiving morphine 10mg/kg, however, displayed no apparent changes in behaviour.

Formalin test results

The overall results demonstrated that there were significant ($p<0.05$) 'within subject' (i.e. the differences in time variable for each group) and 'between subject' (i.e. the differences between groups) effects as shown by repeated measures ANOVA. This meanst that significant differences exist in the formalin test score over time and the pattern of changes is not the same for the non-stressed and stressed groups as well as the different treatment groups.

Formalin test results in non-stressed groups

Formalin produced the typical biphasic pain response in the saline group (Figure 1). The first phase includes a burst of activity within 30 seconds of formalin injection. This phase lasted for about 5 minutes and was followed by a 5 to 10 minutes of reduced response i.e. the rats showed very little nociceptive behaviour, and then by a second phase of activity that lasts for at least 60 minutes after the formalin injection.

For both the morphine and ketamine groups of rats, the biphasic response was markedly attenuated compared to the saline group signifying analgesia. This attenuation was marked at 10 minutes until 35 minutes post-formalin injection, after which the formalin scores for both treatment groups started to increase. From the graph, morphine showed greater analgesic effect compared to ketamine although comparison between morphine and ketamine groups did not show significant differences except for one instance at 40 minutes post-formalin.

Formalin test results in stressed groups

For the stressed groups, morphine and saline groups showed biphasic pattern but the second phase of the formalin test was depressed (Figure 2). While for the ketamine group, the second phase was completely suppressed, obliterating the biphasic pattern. At 5 minutes post-formalin, which is equivalent to phase 1, ketamine demonstrated the lowest score which was significantly ($p<0.05$) lower than saline. After 5 minutes, however, all three groups showed marked attenuation of the formalin score. For saline this attenuation lasted until 35 minutes post-formalin when the score started to steadily increase again. Morphine showed slight increase in the score after 35 minutes but the scores were still significantly ($p<0.01$) lower than the saline group. Ketamine, however, demonstrated the greatest analgesia by complete attenuation of the formalin score until 60 minutes post-formalin.

Formalin test results during phase 1

Comparing both the non-stressed and the stressed groups during phase 1 (5 minutes post-formalin injection), there were no significant differences among the three non-stressed groups. Among the stressed groups, the only significant difference was between saline stressed and ketamine stressed ($p<0.05$) (Figure 3).

Analyzing the formalin scores for each drug, it was demonstrated that swim stress did not cause any significant differences in the formalin scores for saline, morphine and ketamine, i.e. there were no significant differences between the formalin scores of non-stressed and stressed groups during phase 1.

Formalin test results during phase 2

During phase 2 (10 to 60 minutes post-formalin injection), among the non- stressed groups, both morphine and ketamine showed significantly lower formalin scores than saline ($p<0.001$) indicating analgesia (Figure 4). All the stressed groups, including saline stressed, had significantly lower scores when compared to the saline non-stressed.

Among the stressed groups, both morphine stressed and ketamine stressed showed significantly lower scores than saline stressed (morphine: $p<0.01$; ketamine: $p<0.001$). No significant differences were seen between morphine stressed and non-stressed. Formalin score for ketamine stressed was significantly lower than ketamine non-stressed ($p<0.05$) and saline stressed ($p<0.001$). No significant differences were seen between ketamine stressed and morphine stressed.

Summary of formalin test results

In the non-stressed groups, both morphine 10mg/kg and ketamine 5mg/kg produced analgesia in the formalin test. All three stressed groups demonstrated stress-induced analgesia during phase 2. This analgesia was enhanced by prior treatment with morphine or ketamine. In the morphine group, stress did not enhance the analgesia produced by morphine alone. In the ketamine group, stress significantly enhanced the analgesia produced by ketamine alone.

Discussion

The biphasic response due to formalin injection as shown by previous studies (27*;* 40; 41; 42) was reproducible in this study. Formalin test was used because it provides a valid model for clinical pain (43). The first phase of the formalin test is due to direct chemical stimulation of nociceptors (27) and involved substance P and bradykinin (44). The second phase involved local inflammatory processes (44) as well as processes in the spinal cord (17).

The finding that ketamine inhibited phase two but not phase one of the formalin test in this study is consistent with previous studies that used even higher doses of ketamine (45). Lee & Lee (46) demonstrated suppression of both phases but the quantification of pain behaviour was only by

counting the incidence of flinching. This study shows that a ketamine dose as low as 5mg/kg is antinociceptive in the rat formalin test. This is consistent with the findings from previous studies (47; 46). Studies done with other NMDA antagonists such as dextromethorphan and memantine (45) and MK-801 (48) also showed similar pattern of Phase 2 inhibition. The fact that ketamine produced preemptive analgesia by preventing central sensitization during Phase 1 as shown by Gilron et al (47) is supported by clinical data suggesting preemptive analgesia with ketamine (5, 49), by electrophysiological study demonstrating inhibition of dorsal horn neuronal firing by ketamine after noxious stimulation (50), and by another behavioural study in a different model of persistent pain (51).

Following systemic administration of ketamine, several mechanisms have been proposed to be involved in producing the analgesia. The first one reflects actions on mechanisms within the spinal cord involving central sensitization (52). Other mechanisms include supraspinal actions, either by inhibiting NMDA receptors at, for example, thalamic sites (54), or activation of descending pain inhibitory mechanisms involving biogenic amines (54). Active metabolites such as norketamine also contribute to systemic actions of ketamine (55). It has also been shown that antagonists of NMDA receptors modulate elevated discharge of spinal nociceptive dorsal horn neurons that manifests as suppression of the second phase of the formalin test (28). Benrath et al (56), in an *in vivo* experiment, demonstrated that low-dose S(+)-ketamine does not affect C-fibre-evoked potentials alone but blocks long term potentiation induction in pain pathways. Long term potentiation was one of the resulting effects of central sensitization whereby there was long lasting increase in the efficacy of synaptic transmission (3).

Swim stress, as expected, reduced formalin nociceptive response during the second phase. Previous studies using similar swim stress paradigm also produced similar result (40). The neuroanatomical locus underlying this opioid-mediated stress-induced response has been shown to be the ventral tegmental area which has both μ and δ receptors (57).

The analgesia produced by this swim stress paradigm has been shown to be mediated by δ-opioid receptor (40). However another study by Vaccarino et al (30) showed that subjecting mice to the same swim-stress paradigm produced a non-opioid analgesia in the formalin test. These researchers demonstrated that another NMDA antagonist, MK-801 (dizocilpine maleate), blocked the analgesia produced by swim stress. Another more recent study also demonstrated blockade of stress-induced analgesia by MK-801 (33). This is in contrast with this study which showed enhancement of stress-induced analgesia by ketamine. However, Vaccarino et al (30) only measured formalin-induced nociceptive response during the initial 10 minutes following formalin injection i.e. equivalent to the first phase. Therefore, the NMDA mediation of the swim stress may be involved only during the first phase. However, in this study, ketamine inhibited the first phase after swim stress i.e. producing analgesia instead of blocking it so some other explanation may be likely for this discrepancy (40). Deutsch et al (58) proposed that swim stress altered or diminished NMDA-mediated neural transmission. Further studies are needed to look at the molecular mechanism that results following administration of ketamine such as determining the expression of c-fos gene, which is mediated through the NMDA receptor.

In conclusion, this study provides evidence that low dose ketamine is antinociceptive in the rat formalin test and this antinociception is enhanced by swim stress. Taking the finding further into the clinical setting, it suggests that under stressful situations such as operative stress, ketamine is capable of producing profound analgesia at a subanaesthetic dose (59). Further studies need to be done to determine the underlying mechanism for this synergistic effect of ketamine and stress-induced analgesia.

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Corresponding Author :

Dr Asma Hayati Ahmad MBBS, MSc (Physiology)
Department of Physiology
School of Medical Sciences
Universiti Sains Malaysia, Health Campus,
16150 Kubang Kerian, Kelantan, Malaysia
Tel: + 609 766 4908
Fax: + 609766 3370
Email: asmahayatiahmad@yahoo.co.uk

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