AN INVESTIGATION INTO THE ROLE OF INSULIN IN PAIN THRESHOLD

THESIS

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In partial fulfillment of the requirements for the award of degree of

DOCTOR OF PHILOSOPHY IN PHARMACY

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I hereby declare that the Ph.D thesis entitled **"An Investigation into the role of insulin in pain threshold"** submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai, is a record of independent work carried out in the Department of Pharmaceutics, Swamy Vivekanandha College of Pharmacy, Tiruchengode under the direct supervision and guidance of **Dr.N.N.Rajendran**, M.Pharm., Ph.D., for the partial fulfillment for the degree of **DOCTOR OF PHILOSOPHY.** This work is original and has not been submitted earlier for the award of any other degree of this or any other university.

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- **B.Nandhakumar**, N.N.Rajendran. Association between endogenous insulin and pain threshold and its interaction with diurnal rhythm in mice on chemical nociceptive tests. Life Sciences (Communicated).

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LIST OF ABBREVIATION

ATP	…	Adenosine triphosphate
DAMGO	…	D-Ala2-NMePhe4-Gly-ol-enkephalin
DAMME	…	D – Ala2, MePhe 4, Met (0) ol
ELISA	…	Enzyme linked immunosorbent assay
G∞i	…	G protein alpha inhibitory
GLUT	…	Glucose transporter type 4
IGF	…	Insulin growth factor
IL 1	…	Inteleukin 1
IL 6	…	Interleukin 6
ISR	...	Insulin secretion rate
K_{ATP}	…	Potassium adenosine triphosphate
L-D Schedule	…	Light – dark
L-L Schedule	…	Light – Light
L – NAME	…	LevoNitroarginine methyl ester
MEK	…	Met enkephalin
MEAP	…	Met 5 enkephalinarg 6 – Phe 7
mRNA	…	Messenger Ribonuceic acid
NO	…	Nitric oxide
NMDA	…	N- methyl D- aspartate

NSAIDs	…	Non-steroidalanti-inflammatory drugs
NLX	…	Naloxone
RA	…	Rheumatoid arthritis
RBCs –	…	Red blood corpusles
RNA	…	Ribonucleic acid
RT-PCR	…	Reverse transcription polymerase chain reaction
SCN	…	Suprachiasmatic nucleus
S.E.M.	…	Standard Error Mean
STZ	…	Streptozotocin
WHO	…	World Health Organization

CONTENTS

1. INTRODUCTION

Pain is part of our life, existing throughout human development, from birth to death. It is a perception triggered in the nervous system and evoked as a result of external stimuli, disease or tissue damage. Pain is important for survival because it acts as protective and alarm mechanism. According to the International Association for the Study of Pain, it is an unpleasant sensory and emotional experience associated with potential or actual tissue damage.[1]It is associated with various diseases, inflammatory conditions, tissue damage and surgical interventions. Various factors such as physiological, pathological and psychological are involved in pain perception. In addition to these factors, various chemical substances are involved in the modulation and transduction of pain such as 5-hydroxytryptamine, gamma amino butyric acid, acetylcholine, histamine, bradykinin, substance P, opioid peptides etc.[2, 3]

In addition, insulin hormone induced hypoglycemia has been reported for its role in antinociception and following this finding, studies were carried out to examine this.[4] The antinociceptive action of morphine is potentiated by exogenous insulin in experimental pain models.[4] Exogenous insulin has inherent antinociceptive action and acts as a neuromodulator.[5,6] Insulin potentiates the antinociceptive effects of sodium salicylates[4] and morphine[8] and its antinociceptive action is mediated through 5-hydroxytryptamine, dopamine and opioidergic receptors.[5] Streptozotocin-induced diabetic mice and rats as well as genetically diabetic db/db mice are significantly less sensitive to the antinociceptive effect of morphine in tail flick test indicating that blood glucose level affect pain perception mechanisms.[9,10] However,

in another similar study in formalin test, the authors propose that hyperinsulinemia in db/db mice might have potentiated the analgesic effect of morphine and compensated for the attenuated pain perception mechanism due to hyperglycemia.[5] A clinical study in praderwilli syndrome patients showed that serum insulin was higher when pain threshold was higher.[11] The normal subjects with higher insulin levels also showed elevated pain threshold for thermal nociceptive stimuli.[12]

While studies support the role of insulin in the modulation of pain, it was not clear whether the antinociceptive action of insulin is dependent on or independent of the glycemic status. To examine this, the mice were subjected to nociceptive stimuli under different glycemic status via euglycemic, hypoglycemic and hyperglycemic and the authors observed the association between serum insulin level and pain threshold which is independent of the glycemic status. The authors also claim that antinociceptive response of flavone, the opioid analgesic is potentiated with increase in serum insulin level by different manoeuvres treatment.[13] Thus it is evident that endogenous insulin may also be involved in the modulation of pain in addition to other chemical mediators.

The mechanisms underlying the involvement of endogenous insulin in pain threshold are not clearly understood. Studies support the presence of betaendorphins in pancreas which is implicated in the neuroendocrine control on islet hormone release. Beta endorphins being opioid peptide influence the secretion of insulin in humans[14] and also in many animal species.[15,16] As opioid analgesics mimic endogenous opioid peptide, they influence insulin release from pancreatic islets.[17] Besides opioid receptors are present in humans as well as in animal pancreatic

islets.[18] Administration of beta endorphins potentiates insulin secretion via common beta cell opioid receptor and that beta-endorphin may exert a paracrine control of insulin secretion.[19] It has also been reported that beta-Endorphin has potent antinociceptive action in rats and its actions are blocked by the specific opiate antagonist, naloxone hydrochloride. The combination of low dose naloxone and pentazocine is reported to produce profound analgesia than that of high dose morphine in animals and in humans and its mechanism and the role of endogenous insulin is unclear.

Many studies report circadian variation in pain threshold and in the neurochemistry of pain as well as in the effects of analgesic drugs. The reason for the variation in pain intensity is due to the magnitude of expression of various mediators such as bradykinin, endorphins etc. As an example, analgesic effect of morphine in mice using hot plate is greatest due to highest expression of enkephalins in the activity period whereas minimal analgesic effect is obtained due to lesser expression of enkephalins during the rest period. Plasma insulin concentration is also dependant on circadian rhythm in several mammalian species. In man, insulin secretion is high during the day whereas insulin secretion is decreased during the night. In animals, insulin secretion is high during the activity period and low during the rest period.

Thus, from the above mentioned literature, it is understood that the role of endogenous insulin in pain threshold, though reported, has not been investigated in detail using different nociceptive stimuli. Besides that, the effect of diurnal rhythm on the association of endogenous insulin with pain threshold has also not been

attempted so far according to our knowledge. Furthermore, endogenous insulin has been implicated in opioid mediated antinociception. Based on the above considerations, the present study aimed to investigate the relation between endogenous insulin and pain threshold and how it is influenced by diurnal rhythm in mice using different pain models. We included in the study pentazocine, the opioid analgesic as a model drug to investigate the aim of the study.

The findings of the present study may help to understand new knowledge about mechanisms of pain, in particular, the chronobiology of pain perception and the implication of endogenous insulin involvement in the modulation of pain that may contribute to newer approaches in the clinical management of pain.

2. REVIEW OF LITERATURE

2.1. Pain

The word 'pain' is derived from the Latin term 'poena' meaning a penalty or punishment. Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage. It acts as biological safety mechanism because pain offers a warning signal when any abnormalities exist in the living biological system. Pain is a major and inevitable health care problem and most commonly perceived symptom.[28] Millions of people worldwide suffer from pain following surgery as a result of unsuccessful acute pain management.[29] It is a personal and subjective, multidimensional experience, and its intensity varies according to various psychological, physiological, social and cultural factors.[28] Untreated pain has the ability to produce acute neurohumoral changes, neuronal remodeling, and long-lasting psychological and emotional distress, and may lead to chronic painful condition. Chronic pain has a harmful effect on physical health, employment, day to day activities, psychological health and socio-economic wellbeing.[30]

2.2. Nociceptors and pain perception

Nociceptors are the free nerve endings of nerve fibers. These fibers are activated specifically by painful stimuli. The noxious information is transformed into an electrical signal and transmitted from the peripheral area to the central nervous system along axons. There are two different types of nociceptors such as high-threshold mechanoreceptors, which respond to mechanical deformation and

polymodalnociceptors, which respond to tissue-damaging factors such as protons, cytokines, 5-hydoxytryptamine, histamine, bradykinin, prostaglandins and leukotrienes. These inflammatory mediators bathe the nociceptors, activating and sensitizing them.[31]

There are two main fibre types namely A Delta and C fibers present in nociceptors. A- Delta fibers are myelinated and produce fast pain. This type of pain is well localized, sharp and stinging in nature. C fibers are unmyelinated and produce slow pain, which is poorly localized and dull and aching in nature. These primary afferent nerve fibers have cell bodies in either the dorsal root ganglia or trigeminal ganglion and terminate in the dorsal horn of the spinal cord. Most enter the dorsal horn in the ventro-lateral bundle of the dorsal root. Once they have entered the spinal cord, the nerve roots may bifurcate into ascending and descending branches, which can enter the dorsal horn one or two segments higher or lower than the segment of origin (Figure 1). There are four basic processes involved in nociception. They are transduction, transmission, perception and modulation of pain. [31]

2.2.1. Transduction of pain

Transduction of pain begins at the periphery. In peripheral areas, primary afferent neurones or nociceptors are distributed. The nociceptors respond to noxious stimuli. Tissue damage and inflammation occur when the free nerve endings of C fibers and A-delta fibers are exposed to noxious stimuli. This leads to the release of excitatory neurotransmitters which sensitize the nociceptors to the noxious stimuli, so initiating the nociceptors to transmit a pain impulse along the C and A-delta

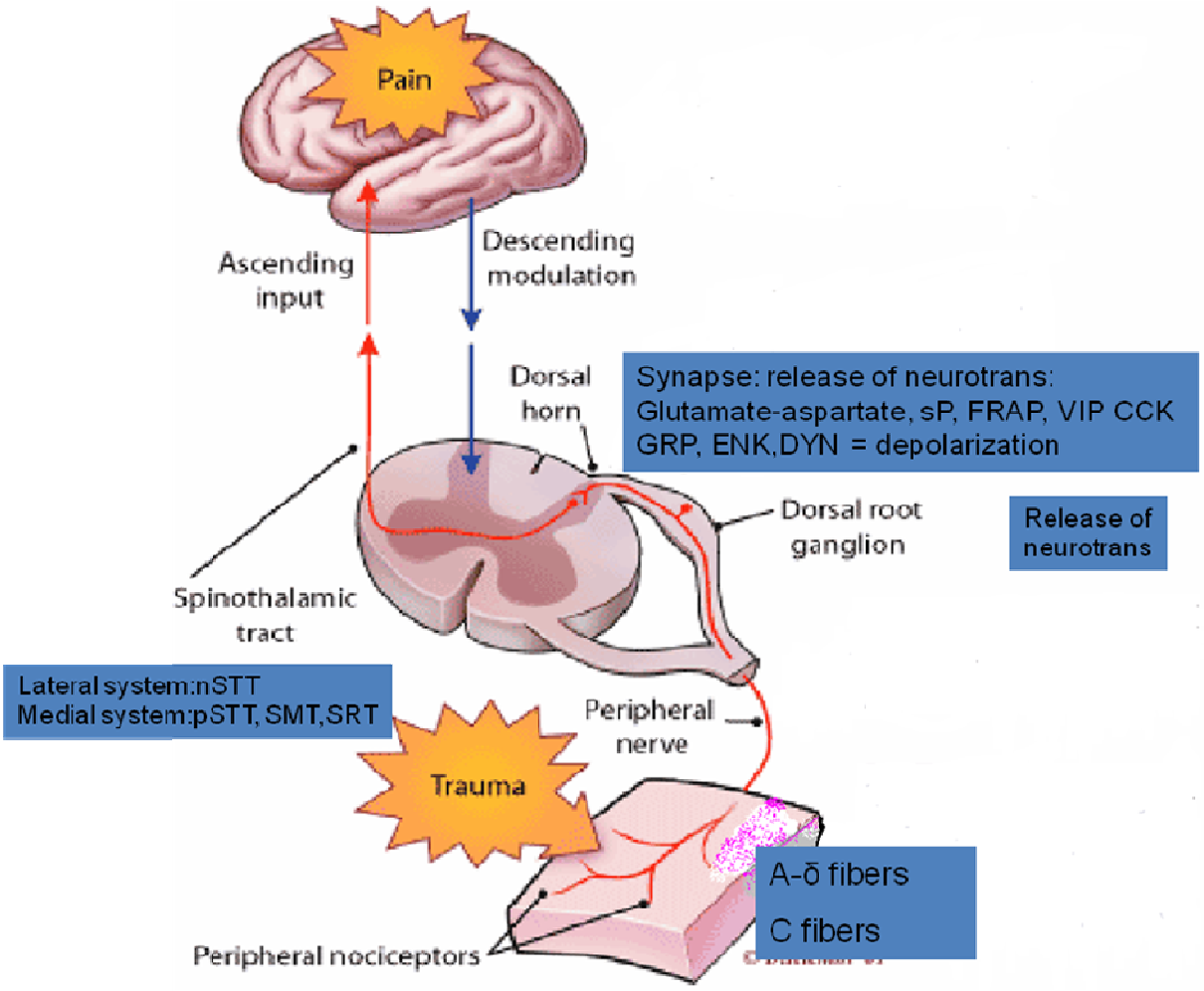


Figure 1: Diagram depicting the major pathways for pain sensation.

fibers. An exchange of sodium and potassium ions occurs at the cell membranes of the C and A-delta fibers which results in an action potential and the transmission of a pain impulse. This ends the process of transduction. [31]

2.2.2. Transmission of pain

The C fibres and A-delta fibers transmit the pain impulse from the site of transduction to the dorsal horn neurones in the spinal cord and the pain impulse is then transmitted from the spinal cord to the brain stem and thalamus via the spinothalamic tract. [31]

2.2.3. Perception of pain

The perception of pain is the result of the neuronal activity of pain transmission. A number of biological structures are involved in the perception of pain. These include the:

Reticular system: This system is responsible for the autonomic and motor response to pain.

Somatosensory cortex: It is involved with the perception and interpretation of sensations. It identifies the intensity, type and location of the pain and relates the sensation to past experiences and cognitive activities.

Limbic system: This system is responsible for the emotional and behavioral responses to pain. [31]

2.2.4. Modulation of pain

The modulation of pain involves changing or inhibiting the pain impulses. The pathways involved in modulation are referred to as the descending pain system, as they involve fibers originating in the brain stem which descend to the spinal cord. The multiple, complex pathways involved in the modulation of pain are referred to as the descending modulatory pain pathways and these can lead to either an increase in the transmission of pain impulses (excitatory) or a decrease in transmission (inhibition).Descending inhibition leads to the release of inhibitory neurotransmitters that block or partially block the transmission of pain impulses, and therefore produce analgesia. Inhibitory neurotransmitters involved with the modulation of pain include endogenous opioids, serotonin, gamma-aminobutyric acid,norepinephirine, neurotensin, acetylcholine and oxytocin. These inhibitory neurotransmitters inhibit the transmission of pain impulses, and therefore produce analgesia. This process is termed endogenous pain modulation. Endogenous opioids namely enkephalins and endorphins are found throughout the central nervous system and prevent the release of some of the excitatory neurotransmitters, therefore inhibiting the transmission of pain impulses and producing analgesia. [31]

2.3. Modulators of pain

Pain perception in humans as well as in animals is modulated by chemical substances such as histamine, bradykinin, acetylcholine, leukotrienes, and prostaglandins. These substances are released into the extracellular tissue when tissue is damaged and pain is perceived.[32] In addition to that neuropeptides such as substance P, neurokininA, neurokinin B, and calcitonin-gene related peptide are

released from sensory afferent nerves. They are pro-inflammatory mediators and cause vasodilatation, plasma extravasation and mast cell degranulation, which result in the release of other inflammatory mediators. They produce hyperalgesia at the peripheral and spinal cord level.[33]

Pain is controlled by different classes of analgesics such as narcotic and non-narcotic analgesics. Narcotic analgesics act by binding to specific opioid receptors in the central nervous system to produce effects that mimic the action of endogenous peptide neurotransmitters. Analgesic properties of opioids are primarily mediated by the mu receptors. Though different classes of drugs such as opioid analgesics, non-steroidal anti-inflammatory drugs in different type of formulations and new drug delivery systems are being used in current clinical practice to ameliorate acute and chronic pain, pain treatment is still insufficient.[2] Therefore different tactic is needed nowadays to assuage the pain intensity in different types of pain.

2.3.1. Opioid peptides

Opioid peptides such as endorphins, enkephalins, dynorphins and endomorphins are produced in the body. Each opioid peptide family derives from a distinct precursor protein and has a typical anatomical distribution. The precursors, prepro-opiomelanocortin, preproenkephalin and preprodynorphin are encoded by three corresponding genes code for the enkephalins, endorphins and dynorphins respectively.[34, 35, 36]

2.3.1.1. Endorphins

Endorphins are endogenous opioid polypeptide compounds. They are produced by the pituitary gland and the hypothalamus during vigorous exercise excitement, pain and orgasm .[37] They are located in more than twenty different parts in the body, such as the pituitary glands as well as in many parts of the brain and nervous system.[38, 39] Four types of endorphins are produced in the human body. They are named alpha, beta, gamma and sigma endorphins.[40]

2.3.1.2. Enkephalins

Enkephalins are pentapeptides involved in regulating nociception in the body. There are two types of enkephalins. They are Met-enkephalins and Leu-enkephalins. Enkephalins are endogenous opioide peptide neurotransmitter found in the brains of many animals, including humans. Leu-enkephalins produce its action through both mu and delta receptors.[41]

2.3.1.3. Dynorphins

Dynorphins are produced from the precursor protein prodynorphin. Prodynorphin is cleaved byproproteinconvertase 2 and multiple active peptides are released. These peptides are namely dynorphinA and dynorphin B. Dynorphins are produced in many different parts of the brain, including hypothalamus, hippocampus, midbrain, medulla, pons and the spinal cord and has many different physiological actions**,** depending upon their site of production.[42]

2.4. Opioid peptides in pain

Opioid peptides and its role in pain in humans and animals have been reported. The endogenous opioid system is an important innate pain-relieving system. This system consists of widely scattered neurons that produce three opioids: beta-endorphin, the met- and leu-enkephalins, and the dynorphins. These opioids act as neurotransmitters and neuromodulators at mu, delta, and kappa receptors and produce analgesia.[43] Pain-suppressing action is mediated in part by endogenous opiate like compounds. This pain suppression system is organized at three levels of the neuraxis: midbrain, medulla, and spinal cord.[44] Like opiates, endorphinsalso have thesimilar abilities to produce analgesia and a sense of well-being. Endorphins act as natural pain relievers.[40] Beta-endorphin and other peptides act as strong peripheral analgesic in the hyperalgesia caused by inflammation.[45]

A study was performed to evaluate the antinociceptive effects of beta-endorphin using tail-flick and hot-plate method. The responses produced by low, intermediate and high intensities of heat stimulation were measured in mice. The results reveal the different neural mechanisms of antinociceptive action of beta-endorphin. These results also led to the suggestion that endorphins are involved in antinociceptive action.[46] Clinical study showed that pentapeptides have potent opiate agonistic activity in brain.[47]

More endorphins are released in the pituitary gland during stress or pain. Exercise promotes the endorphin release too. Beta endorphins are the most potent endogenous opioid neurotransmitters and are found in the neurons of both the central and peripheral nervous system. They are released during pain or stress. The

endorphins produce an analgesic effect during severe pain in our body. Endorphins produce its effect through opiate receptors. Beta-endorphins have the highest affinity for the μ -opioid receptor, slightly lower affinity for the μ and delta opioid receptors and low affinity for the kappa opioid receptors.[40]

It is reported that the opiate-like peptide beta-endorphin and adrenocorticotropin are concomitantly secreted in increased amounts by the adenohypophysis in response to acute stress.[48] A study reported that secretion of betaendorphin from anterior lobe is activated by stress. The rate of translation of the beta endorphin precursor proopiomelanocortin appears to be accelerated by 50% and the rate of conversion of the precursor into products is doubled immediately after an acute stress in animal model. These changes appear to take place at the translational and posttranslational level.[49]

2.5. Pancreatic opioid peptides

Opioid peptides and its presence in the pancreas in humans and animals have been reported. Studies have proved the presence of immunoreactive enkephalin and beta-endorphin in the pancreatic islets has suggested the possibility that opioid peptides may affect the endocrine pancreas by local as well as humoral pathways.[50, 51, 52] Studies indicate that beta endorphin is located in the endocrine pancreas apart from other areas and play the role in the release of islet hormones from pancreas. A study reveals that enkephalin-like immunoreactivity is present in pancreas.[53]

In another study, thin and semithin serial sections of rat pancreas were separated and the presence of endorphins was investigated by using

immunocytochemical staining technique using antisera. The results of the study revealed the presence of small discrete amounts of immunoreactive beta endorphin in the endocrine pancreas and suggest that beta endorphin in the rat endocrine pancreas is implicated in the neuroendocrine control of islet hormone release.[51] Dynorphin is found in the perfusate from rat pancreas.[54] A study reveal that beta endorphin is present in the human pancreatic islets and led to the suggestion that opioid peptides may have effect on the endocrine pancreas by local as well as humoral pathways apart from its the central effects.[52] Another study showed that dynorphins is found in the pancreas.[55]

2.6 Influence of opiates and opioid peptides on insulin secretion

2.6.1. In vitro studies

The influences of the various opiates and opioid peptides on the secretion of the endocrine pancreas have been investigated using different experimental procedures. Beta endorphin and morphine stimulate insulin release from perfused dog pancreas.[16] Similarly, enkephalins Met-enkephalin and Leu-enkephalin stimulate insulin from perfused dog pancreas.[56] It has been reported that betaendorphin inhibit basal insulin release and glucose stimulated insulin release from pancreas of rabbit.[57] Studies indicate that enkephalins in low concentrations appear to stimulate insulin release from isolated rat pancreas[58, 54] and enkephalins in high concentrations seem to inhibit insulin release.[58, 54, 59]

A study in isolated and denervated islet cells showed that various enkephalins inhibit the release of insulin from monolayer pancreatic islet cell culture in a dose

response manner.[60] Morphine stimulate insulin release from perfused dog pancreas.[16] These effects were blocked by naloxone. This result argues in favor of an opiate mechanism of action by these agents.[60] Insulin release by morphine sulphate and low concentrations of enkephalin analogue DAMME from isolated islets of langerhans of the rats were blocked by the specific opiate antagonist naloxone hydrochloride. This led to the suggestion that there may be opiate receptors in islets and that opioid peptides could modulate insulin release.[58]

Dynorphin is a very potent stimulus for insulin secretion and the actions on insulin secretion are not mediated by delta or mu opiate receptors in isolated rat islet of Langerhans.[61]Another study showed that dynorphins is found in the pancreas which also stimulates insulin release from the pancreas.[55] In vitro study using rat islets of Langerhans revealed that the enkephalin analogue DAMME augment insulin release. The potentiatory effect of DAMME on insulin release in the presence of glucose was blocked by naloxone. This study results suggest that insulin release from isolated islets is capable of being influenced by an opioid peptide.[15]

Another study showed that MET- enkephalin stimulates insulin secretion from isolated perfused dog pancreas and the effects of MEK are modulated by the existing glucose concentration. It is also found that morphine stimulates insulin secretion and these effects were blocked by naloxone. The results suggest the entry of enkephalins into the islets by neural pathways and directly modifying insulin secretion in pancreas by interacting with opiate receptors on the islet cells.[56]A study in mouse pancreatic islets incubated under static conditions reveal that alpha 2 adrenoceptor stimulation might inhibit glucose-induced insulin secretion by releasing endogenous

opioids. These effects could be due to mu-opioid receptor activation and opening of K_{ATP} channels.[62]

It has been reported that morphine and beta-endorphin invariably stimulate insulin release from pancreas and the changes brought by morphine and opioid peptide in pancreatic hormone release was blocked by naloxone. This result argues in favor of an opiate mechanism of action by these agents.[16, 60, 54] The results of in vitro studies are inconclusive because the influence of opioid peptides and opioids on pancreatic function appears to be dependent on the agent used, dose administered and concentrations of glucose.

2.6.2. In vivo studies

It has been reported that beta-endorphin andenkephalins increase insulin release in the dog.[16, 56]The pancreatic hormones insulin is elevated by opioids apparently by an action at the islet cells.[63]A study demonstrated that morphine atabout twice the therapeutic dose used in clinical practice, increases insulin secretion without change in blood glucose level in normal dog. However, the same dose of morphine increases plasma glucose level in the absence of accompanying insulin secretion in alloxan diabetic dogs.[64]

Another study demonstrates that enkephalin increase insulin secretion in rats. It is also found that administration of the synthetic human beta endorphin potentiated insulin secretion by the isolated perfused rat pancreas when glucose is present in the perfusate at concentrations of either 125 or 200 mg/dl. This observation led to suggest that beta endorphin potentiate insulin secretion via a

common beta cell opioid receptor and that beta endorphin may exert a paracrine control of insulin secretion.[19]

Beta endorphin inhibits insulin release in the rabbit.[65] Similar results have been reported in the dog with the potent enkephalin anologue DMPE.[66] Another study show that beta-endorphin levels are modulated either within the D cell or upon A and B cells, thereby regulating the secretion of insulin.[67] The effect of intravenous injection of beta-endorphin on insulin release in rats showed the inhibition of insulin release when intravenous injection of beta-endorphin was made.[68]

Dynorphins a kappa receptor agonist found in the pancreas also stimulates insulin release from the pancreas.[55] It has been reported that the widespread presence of opioid peptides and their receptors in brain and periphery and these peptides and receptors correlates with actions produced by opioid agonists and antagonists on hormone secretion. The pancreatic hormones insulin is elevated by opioids by an action at the islet cells.[63] Another study investigated the effects of beta endorphin on basal and stimulated insulinsecretion in the mouse. This study showed that beta endorphin at low dose levels inhibits and at high dose levels augments stimulated insulinsecretion.[69]

2.6.2. Human studies

A study was conducted to examine the influence of opioid peptide on islet function in human beings. The results of the study indicate that peripheral administration of opioid peptide exerts a stimulatory effect on insulin and glucagon

circulating levels. Furthermore, the single intravenous bolus of beta-endorphin elicits a rise in insulin level within 5 min and a parallel increase in plasma glucose.[14]

Synthetic human beta-endorphin was administered intravenously in healthy volunteers and in insulin-dependent diabetic patients. Plasma concentrations of insulin were found to be increased by beta endorphin in nondiabetic subjects. Glucose and insulin responses to beta endorphin could not be blocked by intravenous naloxone. The results suggest that the effect of beta endorphin on islet cell function is relatively resistant to naloxone.[70]Although opioid peptides have been repeatedly reported to influence the secretion of insulin in many animal species, conflicting data have been reported. This might be apparently related to the system used, the species investigated, and the dose administered.[71]

2.7. Insulin

Insulin is a hormone secreted by beta cells in the islets of Langerhans in the pancreas containing 51 amino acids. Insulin is a protein comprising of two polypeptide chains A and B. The A chain is composed of 21 amino acid residues, and the B chain has 30 amino acid residues. Chains A and B are linked by disulphide bridges. C-Chain, which connects A and B chains is liberated along with insulin after breakdown of proinsulin.[72] The molecular mass of insulin is about 5734 daltons. The average daily secretion is about 2 mg or 50 units. The half-life of insulin is about 4 minutes. It is metabolized chiefly in liver and kidneys.[73]

2.7.1. Biosynthesis of Insulin

Insulin is synthesized in the beta cells of pancreas in the form of preproinsulin which is the ultimate precursor and gene for the same is located on chromosome 11. This single-chain precursor consists of A chain and B chain and they are connected by the C peptide. The initial translation product preproinsulin is attached to the N terminus of the B chain. This signal sequence is needed for the translocation of preproinsulin into the rough endoplasmic reticulum. Preproinsulin is cleaved into proinsulin by proteolytic enzymes in the rough endoplasmic reticulum. This proinsulin is then transported to the Golgi complex. The conversion of proinsulin to insulin starts in the golgi complex, continues in the secretory granules, and is nearly complete at the time of secretion. Thus, equimolar amounts of C peptide and insulin are released into the circulation. The C peptide has no known biological function but serves as a useful index of insulin secretion.[74]

2.7.2. Insulin secretion

Insulin is secreted from the beta cells in response to various stimuli like glucose, arginine, sulphonylureas. Physiologically glucose is the major determinant. Various neural, endocrine and pharmacological agents can also exert stimulatory effect. Glucose stimulates insulin secretion through a series of regulatory steps that begin with its transport into the beta cells by the GLUT 2 transporter. Further metabolism of glucose via glucose- 6- phosphate and pyruvate generates ATP which inhibits the activity of ATP sensitive potassium channels. The inhibition of this potassium channel induces opening of voltage-dependent calcium channels and stimulation of insulin secretion.[75]

Insulin promotes glucose entry across cell membrane into the cells of heart, muscle, adipose tissue and all other tissues except brain, liver, renal tubules, intestinal mucosa, islet cells and RBCs. Insulin increases peripheral utilization of glucose, increases glycogenesis, decreases gluconeogenesis and favours conversion of glucose to fat. Insulin promotes protein anabolism and inhibits protein catabolism in liver, muscle and other cells. It also has a protein sparing action. Insulin promotes lipogenesis, inhibits lipolysis. Insulin promotes potassium and phosphate entry into cell. Insulin reduces sodium uptake.[76]

2.7.3. Regulation of Insulin Secretion.

Insulin secretion is regulated by concentrations of glucose in blood. This regulation is achieved by various nutrients, gastrointestinal hormones, pancreatic hormones, and autonomic neurotransmitters. Glucose, amino acids, fatty acids, and ketone bodies promote the secretion of insulin. The islets of Langerhans are richly innervated by both adrenergic and cholinergic nerves. Stimulation of alpha 2 adrenergic receptors inhibits insulin secretion, whereas alpha 2 adrenergic receptor agonists and vagal nerve stimulation enhance release. Glucose is the principal stimulus to insulin secretion in human beings and is an essential factor for the actions of many other secretagogues.[77] The sugar is more effective in provoking insulin secretion when taken orally than when administered intravenously because the ingestion of glucose induces the release of gastrointestinal hormones and stimulates vagal activity.

Several gastrointestinal hormones promote the secretion of insulin. The most potent of these are gastrointestinal inhibitory peptide and glucagon like peptide 1. Insulin release is also stimulated by gastrin, secretin, cholecystokinin, vasoactive

intestinal peptide, gastrin releasing peptide and enteroglucagon. When insulin is evoked by glucose, insulin secretion is biphasic: The first phase reaches a peak after 1 to 2 minutes and is short-lived; the second phase has a delayed onset but a longer duration. The half-life of insulin in plasma is about 5 to 6 minutes in normal subjects and patients with uncomplicated diabetes.[78]

2.7.4. Insulin receptors

Insulin initiates its actions by binding to a receptor. Insulin receptors are present in liver, muscle, fat, circulating blood cells, neurons, and gonadal cells. The insulin receptor is a large transmembrane glycoprotein composed of two 135,000-dalton alpha subunits and two 95,000-dalton beta subunits. The subunits are linked by disulfide bonds.[79]After insulin is bound, receptors aggregate and are internalized rapidly and undergo receptor dimerization. This initiates signal transduction. After internalization, the receptor may be degraded or recycled back to the cell surface. Insulin receptors are present in all cell membranes including those which do not need insulin for glucose entry (eg, Brain). There is an inverse relationship between insulin receptors and plasma insulin. Excess insulin decreases and low insulin increases the number of insulin receptors.[73]

2.7.5. Brain insulin

Studies report that Insulin and insulin-related peptides are produced by various endocrine cell types throughout the animal kingdom.[80, 81,82] It has also been reported that insulin and insulin related peptides peptides are also produced in the central nervous system of vertebrates and invertebrates.[83, 84, 85] Further studies in this

line reveal that the brain has specific receptors for insulin which are widely distributed and appear to be on neuronal and nonneuronal elements. [86-88]

Insulin has been found in the brain, however, the source and actual concentration in nervous tissue is controversial. The presence of insulin and insulin receptors in brain suggest a functional role in central nervous system. Though insulin receptors in the brain and peripheral tissues have same pharmacological properties, these receptors differ in regulation by insulin and molecular size.[86, 87] A study showed that structural differences, including molecular weight, antigenicity, and carbohydrate composition exist between insulin receptors in brain and peripheral target tissues.[89]

Another study results also suggest that functional insulin receptors are present in the brain during development in the rat and the structural differences exist between neuronal and glial cell and between brain and nonneuronal insulin receptors.[90] While it was long considered that the brain was insulin-independent, it has now been established that brain insulin plays a crucial role in the regulation of metabolism, and that altered insulin action in the brain is directly involved in metabolic diseases such as obesity, diabetes or the metabolic syndrome.[91]

It has been reported that insulin produced by the pancreatic beta cells and specific insulin receptors are widely distributed in the networks of the central nervous system and they are related to feeding, reproduction or cognition.[92-97,6] Further studies evidence that insulin receptors are present in particularly high concentrations in neurons, and in lower levels in glia. The messenger RNA of

insulin receptors is abundantly localized in neuronal somata and receptor protein is found in both cell bodies and synapses.[97, 98]

It has been stated that the major molecular structure and most of the properties of brain insulin receptors are identical to peripheral insulin receptors.[99] Studies are in favour of the concept that brain insulin is exclusively of pancreatic origin.[97,100] Transport of blood insulin in the brain has been convincingly demonstrated. Insulin can enter into circumventricular regions which lack a blood brain barrier, and can cross the blood brain barrier via insulin receptor-mediated active transport.[101, 102]

Evidences based on cultured neurons and molecular biology approaches are in favour of a de novo local insulin production in the brain. Reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization approaches clearly indicate that insulin and preproinsulin messenger RNA is expressed in the fetal, newborn and the adult rodent brain and has been detected in the hypothalamus, the cortex and the hippocampus.

Brain insulin is 10 times higher than plasma insulin concentrations and brain receptor content, which is equivalent to receptor content on peripheral tissues, appears to be regulated entirely independently of hormone and receptor in the periphery. The results suggest that insulin in the central nervous system is synthesized by the neural elements and plays a role in the central nervous system which is unrelated to peripheral glucose metabolism.[96, 103 – 111]

2.8. Insulin as pain modulator

2.8.1. Pre-clinical studies on insulin and pain threshold

Earlier studies in experimental animals have identified the role of exogenous insulin in the modulation of pain. The analgesic response produced by insulin pretreatment in rats was 30 per cent greater than response to morphine alone. Insulin was found to potentiate and prolong significantly the analgesic action of morphine and thus provides evidence that insulin induced hypoglycemia enhance the antinociceptive action of morphine in the rat tail-flick test.[5] The exogenous insulin potentiates the antinociceptive effect of sodium salicylates.[7] Another study reports that hypoglycemic doses of insulin does not change acetic acid induced writhing in mice. However, insulin attenuates formalin-induced nociceptive responses in dose-dependent manner and these effects are more potent on the second phase.

The antinociceptive activity of insulin in the formalin test is inhibited by naloxone. Intracerebroventricular administration of insulin also produces antinociceptive effect in streptozotocin-induced diabetic mice and genetically diabetic db/db mice; however antinociceptive effect of insulin is lesser than in normal mice. The results suggest that insulin possess an inherent antinociceptive response and insulin attenuates chronic pain rather than acute pains through a mechanism mediated by opioids besides dopamine and 5 hydroxytrptamine. This study also evidence that antinociceptive pathway appears to be deranged by diabetes mellitus[5]which is supported by another study where the blood glucose level affects the morphine analgesic response by affecting cellular energetics.[8]

Further study shows that the antinociceptive activity of morphine is significantly reduced in STZ-induced diabetic mice, but is not changed in genetically diabetic db/db mice in formalin test. Although hyperglycemia is common in both STZ-induced diabetic mice and db/db mice, insulin level are different, the former being hypoinsulinemic, and the latter hyperinsulinemic.[112, 113] In another study, it has been reported that the antinociceptive effect of intrathecal morphine is significantly reduced in diabetic rats and these effects are reversed with insulin replacement.[114] These evidences led to hypothesize that hyperinsulinemia in the db/db mice might have potentiated the analgesic effect of morphine and compensated for the attenuated pain perception mechanism due to hyperglycemia.[5]

However, another study finding conflicts with these findings. The antinociceptive potency of morphine is decreased in spontaneously diabetic mice.[9] The oral antidiabetic agent CS-045 improved the antinociceptive effect of morphine in spontaneously diabetic mice.[115] The oral anti diabetic agent CS-045 also showed decrease in plasma glucose and increase in insulin levels in spontaneously diabetic mice.[116,117] These results seem likely that not only hyperglycemia but also hyperinsulinemia may be responsible for the reduction in the antinociceptive effects of m-opioid receptor agonists in spontaneously diabetic mice.[118]Another study showed that antinociceptive effect of DAMGO was attenuated by insulin in spontaneously diabetic mice and suggest the increased tyrosine kinase activity due to hyperinsulinemia play a role in the reduction of antinociceptive effect of DAMGO in spontaneously diabetic mice.[118] However, role of insulin for the reduction of antinociceptive effect is not clearly established.

Flavone, a known opioid drug, induced changes in serum insulin level independent of the glycemic status in acetic acid induced abdominal constrictions method in mice proposing that there is dissociation between glycemic status and antinociceptive response. The serum insulin level is enhanced independent of the glycemic status in antinociceptive response of flavones using abdominal constrictions method. Similarly, whenever potentiated antinociception is recorded through various manoeuvres, there is significant further enhancement in serum insulin level which is independent of the glycemic status.[13]

A study on the possible mechanisms of insulin antinociception in mice using the tail flick test suggest the role of 5-HT, dopamine, NMDA, opioidergic receptors and potassium and calcium channels in insulin analgesia.[119]Antinociceptive effect of 7-Hydroxy flavones which induce antinociception like morphine do not suggest a cause-effect relationship between the changes in the glycaemic and algesic state and the results further suggest insulin which is controlled by ATP sensitive potassium channel at the cellular level might also modulate antinociception.[120] There is a high incidence of painful neuropathies in normoglycemic or moderately hyperglycemic patients.[121]

Studies on humans and animals suggest that factors other than hyperglycemia may play a role in the development of painful distal peripheral neuropathy.[122-127] A study in a streptozotocin rat model suggests that mechanical hyperalgesia is a sign of distal peripheral neuropathy, is caused by insulinopenia without accompanying hyperglycemia.[127] Many studies also indicate that insulinopenia plays as a factor in development of peripheral neuropathy.[128-130]

Further study reveals that mechanical hyperalgesia can be triggered by moderate insulinopenia irrespective of glycemic status of the animals and the low-dose insulin replacement normalize the pressure threshold without affecting blood glucose level in the streptozotocin rat model that developed hyperglycemia or remained normoglycemic.[131] Systemic or local hyperglycemia can induce mechanical hyperalgesia[132] impaired nerve conduction velocity and nerve blood flow[133] in non-diabetic animals. Insulin has shown been proved to have favorable effects on various manifestations of diabetic neuropathy, such as reduction of hyperalgesia in the formalin test, increase in nerve conduction velocity,[128,134-136] prevention of mitochondrial inner membrane depolarization,[137,136] support neurite growth,[138,139] prevention of peripheral nerve composition and metabolism changes.[140]

The low-dose insulin therapy showed a improvement of sensory and motor nerve conduction velocity, nerve blood flow during a formalin test.[135] Further studies indicate that nerve conduction velocity decreases early after diabetes induction with blood glucose levels above 350 mg/dl and is fully restored by insulin therapy in euglycemia.[133-134] Another study reveals that an insulin deficit with impaired insulin signaling rather than hyperglycemia plays an essential role in the pathophysiology of painful diabetic neuropathy in animal model [141] and impaired peripheral nerve insulin receptor signaling coincides with early mechanical hyperalgesia and thermal hypoalgesia in STZ-diabetic rats.[142] Insulin in combination with hesperidin (a neuroprotective substance) reverses neuropathic pain.[143]

Duration of diabetes is also a factor for the different nociceptive thresholds in diabetic rodents. Hyperalgesia was observed at an early stage of diabetes in the hot plate test whereas hypoalgesia was recorded in a late stage of diabetes.[144] Another study also reported similar changes using the tail flick test.[145] These results suggest that short term diabetes may be associated with thermal hyperalgesia while long-term diabetes is associated with thermal hypoalgesia in rodents.[144, 145] Another study on the role of endogenous opioid peptide mediation of elevated pain threshold in adult male Sprague-Dawley rats with long-term diabetes mellitus induced by streptozotocin support the hypothesis of endogenous opioid peptide mediation of hypoalgesia in chronically diabetic rats that can be prevented by insulin treatment.[146]

The relation between cellular components expression and morphine analgesia in long term diabetic animals and how it is influenced by insulin treatment has been documented. Opioids action are mediated through G protein linked receptors.[147]A significant increase in the mRNA levels of G∞i was observed in the dorsal portion of the lumbar spinal cord in diabetic animals. However, in diabetic animals that received insulin, levels of G∞i mRNA and protein were close to those in control rats.[114]

Basal insulin secretion is partially regulated by the sympatho adrenal system and secretion is species dependent. Endogenous catecholamines have the ability to promote insulin secretion.[148] Alpha2 adrenergic agonist increases pain threshold and potentiate the analgesic effect of opioids both in experimental animals as well as in humans.[149-153] Administration of clonidine to insulin treated diabetics elevated nociceptive threshold. [154] The pancreatic β cell possess α_2-adrenergic receptors on its

plasma membrane to sense the corresponding ligands adrenaline or noradrenaline to govern glucose-stimulated insulin secretion. Alpha 2-adrenergic agonist clonidine concentration-dependently depresses glucose-stimulated insulin secretion.[155] Though central noradrenergic system involves in the regulation of nociception and adrenergic innervations are present in the pancreatic islets in both humans and experimental animals, role of insulin has not been clearly established.

2.8.2. Clinical studies

Clinical studies further evidence the relationship between insulin and pain threshold in patients[11] and normal subjects.[12] Patients with Praderwilli syndrome, obese and non-diabetic people were subjected to heat induced pain. The latency thermal and pain threshold was significantly higher. Blood samples were analyzed and showed that serum insulin was significantly increased.[11]

A study was carried out to examine peripheral sensory nerve function and its dependence on insulin using healthy non-diabetic control subjects, obese individuals and diabetic subjects. The results revealed that insulin has an action on nervous tissue in addition to its effects on glucose metabolism. The mechanisms of this action remain to be elucidated.[12]

2.9. Chronobiology of pain

Pain is difficult and sometimes frustrating to treat. In spite of new devices and new approaches have been developed in recent years, pain management is still in improvement stage. The depth of pain intensity varies from one patient to the next, and there are also some studies suggesting that the intensity of pain varies

according to time of day.[22] Modern pain therapy widely follows the WHO guidelines using a three-step 'ladder' for pain relief. This escalating step scheme includes the administration in the order nonopioids, mild opioids and strong opioids, and adjuvants at any step. Analgesics should be given 'by the clock' rather than 'on demand'. However, the chronobiological parameters time course of pain rhythm, circadian efficacy of analgesics, and individual circadian need for analgesics are to be considered. The results of studies in chronobiology are not consistent. Therefore, further detailed and in depth studies are necessary to analyze consistent rhythms to diseases, pain causes, and analgesic efficacy of opioids.

Many studies reveal that each patient perceives pain differently and its intensity varies individually during the time of day. Fluctuation in pain intensity exists in patients who receive analgesics over a constant time. These kinds of outcomes from many studies in chronobiology prove the influence of biological rhythms on the pharmacokinetic and pharmacodynamics aspects of analgesics. As different types of pain show different rhythms, analgesics should be dosed flexibly. It is also very important that drug therapy can be adjusted individually to the pain rhythm of the patient as well as to the type and cause of pain. A flexible dosage depending on pain intensity and rapid dose adjustment are essentials of a modern pain therapy.[156]

2.10. Circadian rhythms

The rhythms having 24 hour periods are classified as circadian. Biological rhythm synchronizes various behavioral, biochemical and physiological process in plants as well as animals with changes in environmental factors. A circadian rhythm

is generated by an endogenous circadian oscillator. Circadian rhythm are present in neuronal and non-neuronal cells including neuronal cells in the frontal cortex, purkinje cells in the cerebellum, liver cells and fibroblasts. Suprachiasmatic Nucleus (SCN) of the mammalian hypothalamus is a master clock that controls endogenous circadian oscillator (Figure2).A circadian system is a mysterious mechanism, located in the brain of mammals. This is present even in unicellular organisms that functions as a clock. This clock drives circadian rhythms. It is independent but remains responsive to environmental cycles.[157-159]

2.11 Importance of circadian rhythm

Circadian rhythmicity is key in the pathophysiology, diagnosis, and treatment of clinical disease.[160] The existence of internal clocks that control circadian rhythm has been established at molecular level (Figure 3).[161] The circadian rhythm exist not only in the sleep/wake pattern, it also exist in respiration, heartbeat, blood pressure, smooth muscle tone, peristalsis, heart rate, organ functions and nerve activity (Figure 4).[162] Circadian rhythm plays in hormone release, receptor expression, and gene relation (Figure 5).[161] The importance of understanding the circadian variation and circadian regulation of gene expression and how it is affected in disease states may be useful in optimizing dosing time. This approach helps to achieve maximum therapeutic efficacy with minimum toxicity. Hence, circadian rhythmicity can be important in optimization of time of drug administration.

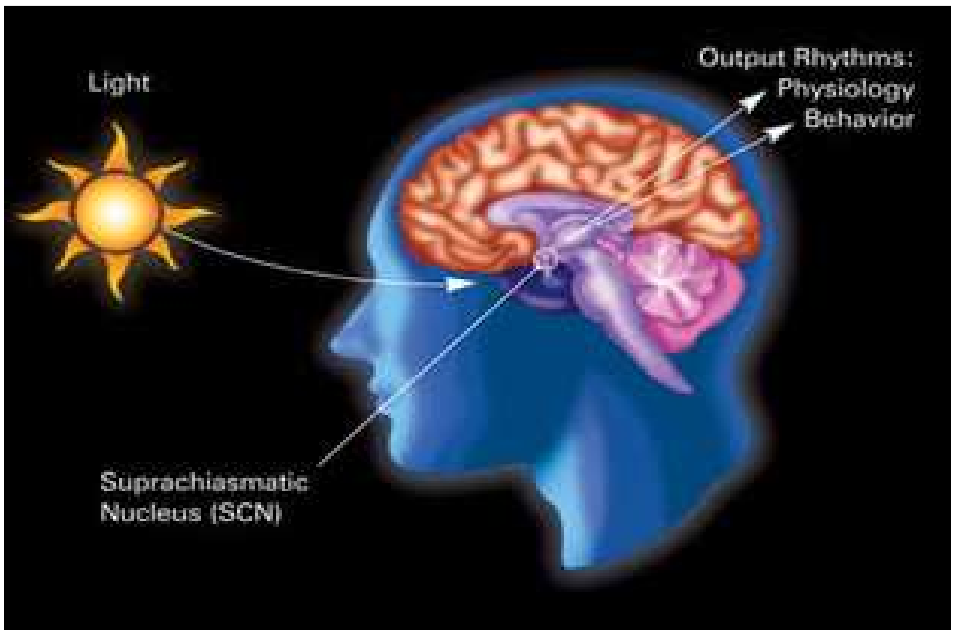


Figure 2: Diagram representing relation between circadian master clock suprachiasmatic nucleus (SCN) and circadian rhythm.

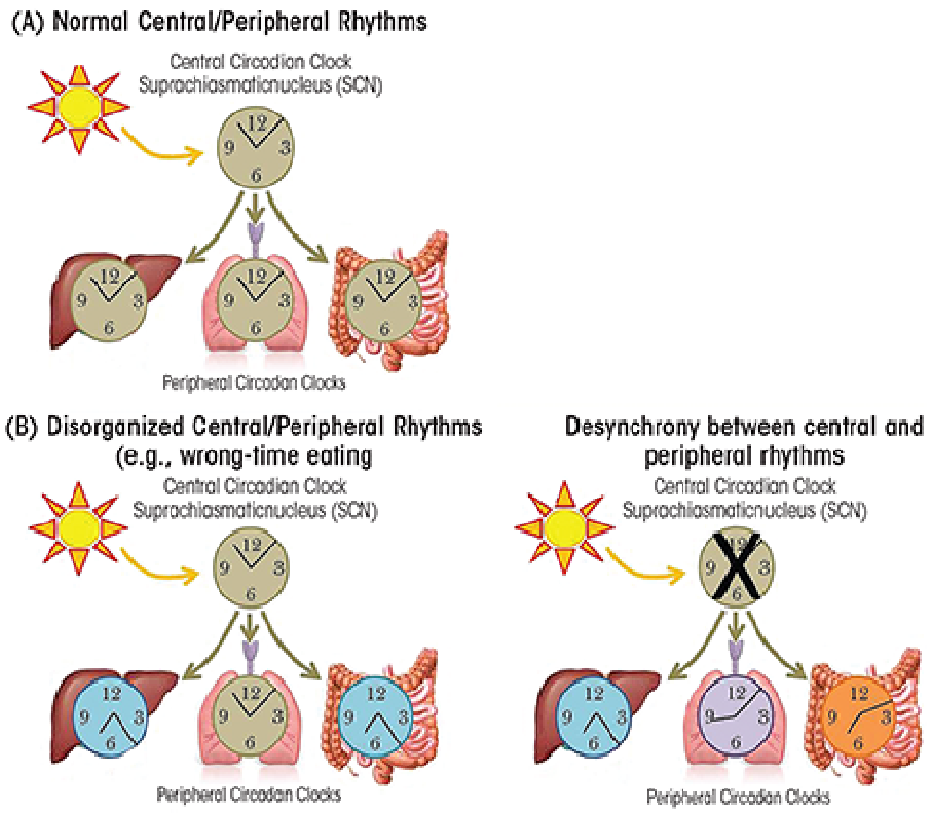


Figure 3: Schematic diagram showing normal (A) and disorganized (B) central and peripheral circadian clocks.

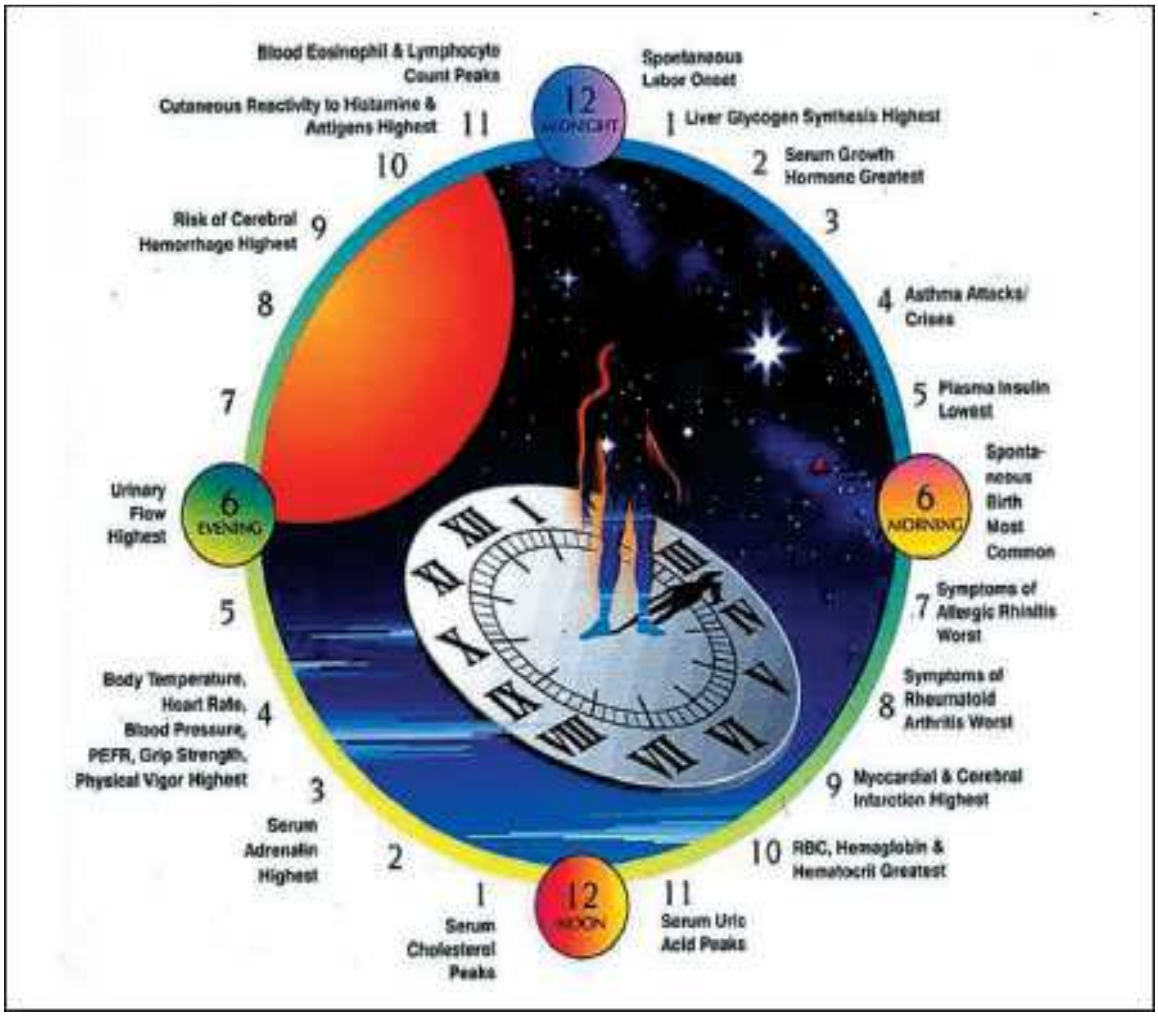


Figure 4: Diagram showing features of the human circadian (24-hour) biological Clock.

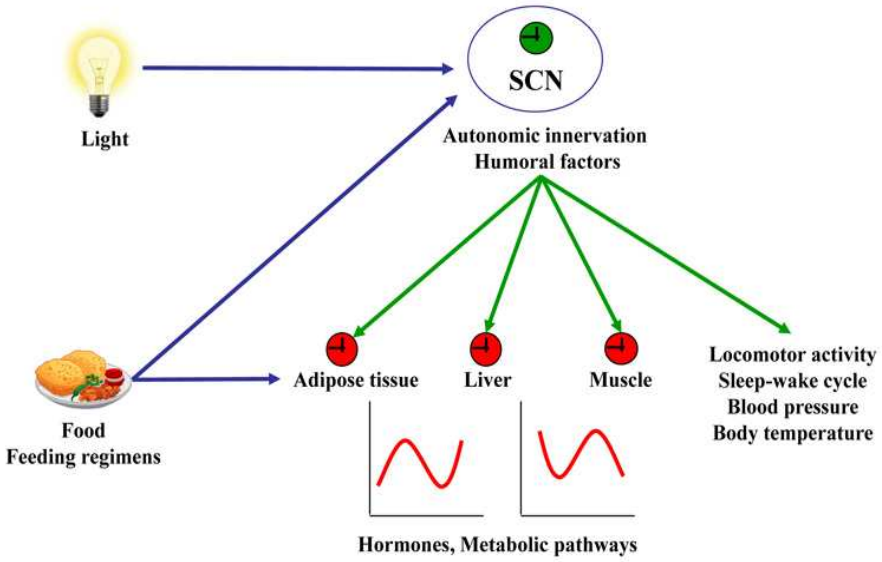


Figure 5 Diagram depicting the effect of circadian rhythm on hormones and metabolic pathways through autonomic innervations.

2.12. Pain threshold and circadian rhythm

2.12.1. Preclinical studies

Many studies have been conducted on laboratory animals to determine the 24-hour variation in pain threshold. Variations in the intensity of pain perception using different animal models have been studied. Using the hot-plate test, it was found that the highest threshold occurred at the end of the resting period, while least threshold was found at the end of the activity period. [163, 164, 3, 23] Circadian rhythm of pain in response to the thermal stimuli was assessed in male mice. The response latencies were measured using the hot plate method. Highest latencies were obtained at 12 and 4 hours whereas minimal latencies were noticed at 10 and 20 h.[165]

The daily variation in an intensity of kaolin-induced writhing reaction was examined in mice kept under conditions of light; 07:00 - 19:00 and dark; 19:00 - 07:00. The number of writhes was counted for 30 minutes after a single intraperitoneal injection of kaolin at 00:00, 02:00, 04:00, 06:00, 08:00, 10:00, 12:00, 14:00, 16:00, 18:00, 20:00 and 22:00. The number of writhes was found to be a peak at 18:00 and a trough at 06:00. These results suggest that the kaolin-induced writhing reaction shows the daily variation with a peak at the end of the resting period and a trough at the end of the active period.[166]

The influence of morphine dosing time on analgesic effect after acute or chronic treatment and their pharmacological mechanisms were investigated in ICR male mice under light and dark cycle. The results showed that there was a significant 24-h rhythm in the latency of thermal response after morphine injection. Latency

was found to be a trough at the light phase and a peak at the dark phase. Especially, at the dark phase, the time spent on the hot-plate after morphine injection was significantly longer compared with non-drugged state. The rhythmic pattern of analgesic effect induced by morphine was similar to that of the sensitivity of mice to painful stimuli in non-drugged state.[167]

A study was conducted in mice to assess the influence of dosing time on pharmacological effects of acetyl salicylic acid under light and dark cycle using hot plate method. In that study, it was found that the mice given saline spent significantly longer latency time during the dark phase than during the light phase. The mice given acetyl salicylic acid also showed a significant circadian rhythm in the time spent on the hot plate. The shortest latency was found at early light phase and the longest latency was observed at mid dark phase.[168]

A study was conducted to evaluate the effect of increasing doses of morphine and nalorphine on total rat brain acetylcholine content. The effects were analysed 30 min after the administration of the drugs at 07.30hr and 19.30hr. The results indicate that the effects of morphine and nalorphine on brain acetylcholine levels is altered depending upon the time of administration. The results suggest that morphine in small dose significantly increased total brain acetyl choline content in the active period whereas a large dose of morphine produces the same effect in the resting period in rats.[169] Another study assessed the changes in pain in relation to time of day and to estrus in female Wistar rats using tail flick method and found that maximal threshold occur early in the activity period of the rats.[170] The animal data suggest the existence of time dependent variations in the pain threshold.

2.12.2. Clinical studies

Pain varies according to circadian rhythm in humans. It is quite important to note that morning pain is found in patients with angina pectoris, myocardial infarction, migraine, rheumatoid arthritis and toothache. Conversely, evening or nighttime pain seems to be more frequent in patients with biliary colic, cancer and intractable pain.[171, 172] Patients having the symptoms of gastro oesophageal reflux disease show night time pain.[173]

The patients with rheumatoid arthritis report the presence of greatest pain in the morning.[174, 175] Joint stiffness and pain are present more in the early morning. One potential cause of rheumatoid arthritis symptoms is the diurnal rhythm of human cytokine production which contributes to inflammation. It has been reported that human cytokine production has peak levels during night and early morning.[175]A study conducted in rheumatoid patients has shown that pain peaks between 6 a.m to 8 a.m and trough at 6 p.m.[174]

A study attempted to determine whether migraine exhibits a circadian rhythm or occurs randomly throughout the day in migraine patients. The results showed circadian variation in migraine onset with a marked increase in attacks between 6 a.m and 8 a.m. These results suggest that alteration of vasomotor tone may be involved in the initiation of migraine attacks.[176]Another study reported that a significant diurnal variation of pain threshold in healthy volunteers using the tourniquet pain model and found pain scores were highest at midnight.[177]

Clinical study was conducted to evaluate biliary pain and time of painful episode in patients with symptomatic gallstones. In that study 50 patients with

symptomatic gallstones were selected. Thirty-eight of the 50 patients showed the time of onset of biliary pain in the 24-h cycle. The time of onset of biliary pain showed significant circadian periodicity with its peak at 00:25 h.[178] It has been studied that chronic pain like cancer pain is peak at 6 pm and trough between 4 am to 10 am whereas acute pain like tooth ache is peak at early afternoon.[179]

Chronobiological studies on humans in relation to pain provide interesting results. A morning peak and/or an evening trough of pain were recorded in healthy subjects using different painful stimuli, such as electrical stimulation, electric shocks, or radiant heat. On the other hand, a nighttime peak was found when pain was induced by an inflatable cuff applied to the forehead for 20 s.[180] It was reported that the rhythmic occurrence of renal colic is peak in the morning, independent of gender and presence or absence of visible kidney stones. It was also been reported that the temporal changes in renal functions result in an increased nocturnal concentration of urine, and this is considered to be a predisposing factor for renal colic attacks in the morning. [181, 182]

Another study was conducted to investigate the occurrence of toothaches at random during the 24 hours and found that toothaches are more in the early morning. The results suggest that the lowest pain threshold of the teeth to electrical stimulation occur during the second part of the night.[172] Another study determined the circadian rhythm of the sensory pain threshold in the teeth in humans. The sensitivity of the teeth was at its nadir at the maximum pain threshold between 15:00 and 18:00h, while the highest tooth pain intensity, together with the lowest pain threshold, occurred at about 08:00h involving a 160% increase. The sensitivity

threshold of the gums to a cold stimulus was at its maximum at 18:00h and at its nadir at about 03:00h.[183.]

An observational study conducted in women with labor pain also follows a chronobiological pain cycle. Circadian variations were assessed in labour pain perception. In that study, 222 consecutive nulliparous women with uncomplicated pregnancy, spontaneous labor, cervical dilatation, ruptured membranes and normal fetal heart rate tracings were studied. Visual analogue pain scores were used for analysis during four periods such as night (1:01 a.m. to 7:00 a.m.), morning (7:01 a.m. to 1:00 p.m.), afternoon (1:01 p.m. to 7:00 p.m.) and evening (7:01 p.m. to 1:00 a.m.). This study reveals that visual analogue pain scores were lower in the morning than in the afternoon, evening and night periods in women with labor pain.[184]

Time dependent rhythms in pain intensity of various medical conditions have been reported. A study assessed whether circadian variation is present in unstable angina or not and also examined the time of onset of ischemic pain in 7,731 patients. The result showed that the onset of pain in the morning hours between 6 a.m. and 12 noon in more number of patients. This circadian variation was observed both in patients with unstable angina. These findings suggest that circadian variation exists in the onset of myocardial ischemic syndromes.[171]

A study was conducted to analyze whether the sudden cardiac death exhibits a circadian rhythm. The authors analyzed the time of day of sudden cardiac death by using death certificates of 2203 individuals. The data reveal circadian variation in sudden cardiac death with a low incidence during the night and an increased incidence from 7 to 11 a.m.[185] Sex hormones also seem to be involved in circadian rhythms of rheumatoid arthritis symptoms. Increased pain intensity and sleep

disturbances are observed during the luteal phase in patients who have rheumatoid arthritis, when estrogen and progesterone levels would be higher than in the follicular phase.[171]

2.13. Circadian variation in the neurochemistry of pain

The circadian rhythmicity of pain perception found in clinical situations and in experimental models of pain seems to be related to the time-dependent variation of chemical substances interacting with nociception. In this regard, several animal and human studies demonstrated circadian patterns in the brain levels of opioid peptides, 5-hydroxytryptamine, bradykinin, glutamate, NO, substance P, cytokines (IL1, IL6) and prostanoids. These chemical agents are involved in the activation of nociceptors and thus may be considered as potential targets for analgesics. For instance, brain concentrations of substance P are circadian time-dependent in rats, with highest values found during their nocturnal activity span.[186]

It has also been found that the high level of metenkephalin during night time is correlated with higher analgesia demonstrated at night in mice.[163] Changes in the opioid peptide levels and circadian variations in the concentration of beta-endorphin have also been revealed in different brain areas of the rat. Beta – endorphin concentrations were found to be peak levels during late in the rest period of the animals.[3] Similarly, two fold higher level of metenkephalin was found in brain of mice in the middle of the activity span than at any other time during the 24-hour period.[186]

A study showed that the beta endorphin, MEAP and MEK changes related to diurnal rhythms in various regions of rat brain and in the pituitary by specific

radioimmunoassay. The results showed that the contents of MEAP, MEK and beta-endorphin were higher in the pituitary of old rats than that of young rats while the content of these opioid peptides was higher in the hypothalamus of young rats than in that of old rats. In the diurnal rhythm study, it was found that both MEAP and MEK contents were higher at mid-dark than at mid-light and that in the intermediate posterior lobe of the pituitary.[187]

Another study reported that the circadian rhythm of beta-endorphin was altered in cancer patients compared to healthy subjects.[188] In human parotid saliva, methionine enkephalin-like, substance P-like and beta-endorphin-like immunoreactivity were shown to be higher in the morning.[189] Melatonin, a chronobiotic hormone could exert an analgesic effect in migraine and cluster headache.[190] Studies in humans also reveal that the levels of endogenous opioid peptides are higher at the beginning of the day and lower in the evening, both in neonates and adults.[191] Melatonin may exert its analgesic action by increasing the release of beta-endorphins rather than by binding to the opioid receptors subtypes.[192]

Animal and human studies reveal that circadian variations can be observed in the pharmacokinetics and effects of NSAIDs. Bioavailability is greatest in the morning. Ketoprofen or indomethacin plasma levels following 7 a.m. ingestion are approximately 50–58% higher than following evening administration.[193, 194] Higher and faster morning absorption has also been documented with controlled release indomethacin and ketoprofen formulations.[195, 196, 197]

2.14. Diurnal variations in analgesic effect

The effects of analgesics are also subject to circadian variation. Studies have been predominantly conducted in animals. A study has demonstrated using the hot plate test that morphine-induced analgesia shows circadian variations. In that study, maximum analgesic effect was obtained in mice in the evening at about 21:00h, during the activity period of these nocturnal animals, whereas the minimum analgesic effect occurred at about 15:00 h during the resting phase.[23]

Another study in this line demonstrated that the response to thermal stimulation and the analgesic effectiveness of morphine during various phases of the diurnal cycle were assessed by the hotplate method. Saline treated controls showed shortest reaction times during the last quarter of the light-phase and first quarter of the dark phase. Longest reaction times were recorded during the last quarter of the dark phase. Doses of 4, 8, 16, and 32 mg/kg of morphine was administered IP at the peak and trough of the pain sensitivity rhythm. Peak analgesic activity was obtained in the group injected during the last quarter of the dark phase while minimal analgesic effectiveness was obtained during the third quarter of the light phase.[198]

A study was conducted to find out the influence of the circadian variation on morphine induced analgesia using the hotplate test in mice. It was observed that morphine-induced analgesia was greatest in the third hour of the animals' activity period; whereas, minimal analgesic effect was obtained in the middle of the animals' rest period.[199] A study using a same model showed the effectiveness of morphine was maximal at the beginning of the rest period. Furthermore, it was also observed that L-NAME, a nitrous oxide synthetase inhibitor, potentiated the effect of

morphine at all hours of day, but this effect was significantly greater during the animals activity period.[200]

The circadian rhythms in the acute toxicity, effects and pharmacokinetics of tramadol were demonstrated in mice.[201] It was found that the mortality was highest when a high dose of the drug was administered in the middle of the animals' activity period. Peak toxicity was obtained when plasma levels were highest. The hot-plate and the tail pressing mouse models were used in this study and it was observed that the analgesia produced by tramadol was greatest at the end of the animals' activity period. In humans, it has been reported that ingestion of 50 mg tramadol in the evening exerted stronger analgesic effects in 18 healthy volunteers.[202]

In another study conducted in postoperative patients undergoing elective cholecystectomies, it was found that the need for fentanyl was lower when the surgery was done between 8 and 10 a.m. than between 11 a.m. and 3 p.m.[203]A study of meperidine reveals a circadian variation of meperidine-induced analgesia in sickle cell anemia patients, with maximal analgesic effect occurring with the morning dose.[204] In yet another study on the circadian variation on the narcotic doses,it was observed that 76% of patients received most of the extra doses of narcotics between 10 a.m. and 10 p.m.; the number of extradoses was 60% lower during the nighttime compared to the daytime.[179]

The effects of a light-dark cycle and of constant light on nociceptive thresholds and morphine-induced analgesia were studied in two strains of mice: C57BL/6 and SEC/1Re. Diurnal rhythm was observed in the responsiveness of mice to nociceptive stimuli, and in the analgesic effects of morphine light-dark cycle.

Under the L-L schedule clear patterns of daily rhythmicity were evident in both strains for both nociceptive thresholds and responsiveness to morphine. Finally, under the L-L schedule, the overall responsiveness to pain and the antinociceptive effects of morphine were found to be increased when compared to the results observed in the L-D condition. The administration of naloxone decreased the nociceptive thresholds. This indicates an involvement of the endogenous opioid peptides.[205]

In an animal study, the influence of morphine dosing time on analgesic effect after acute or chronic treatment and their pharmacological mechanisms were investigated in male mice under a 12-h light/dark cycle. The time-dependent difference in the analgesic effect after chronic treatment is closely related to that in the expression of mu-opioid receptor. This study outcome suggests that 24-h rhythm of morphine analgesic effect is consistent with 24-h rhythm of mu-opioid receptor expression.[167]

Experiments with mice using the hot plate test reported that rhythmic pattern of pain reaction is connected with the increase in endorphin and encephalin. Endorphins and enkephalins are endogenous peptides found in regions of the brain known to be involved in the perception of pain. It was also reported that their brain concentrations fluctuate over 24-hour period. In mice kept on a 12:12 L:D cycle, the levels of MEK in the whole brain of mice were twice as high at the end as at the beginning of the resting period.[206]

Studies were also carried out on hypophysectomized rats and mice in comparison to sham-operated controls in order to assess the role of the pituitary in

the diurnal rhythm in sensitivity to pain, the hyperalgesic effect of naloxone and the effect of stress on brain levels of met-enkephalin. There were no significant differences in jump latencies between hypophysectomized and sham-operated control mice. The jump latencies in the p.m. were significantly greater than those in the a.m. for both the sham and the hypophysectomized mice. In both the sham and hypophysectomized mice and rats, naloxone significantly reduced the jump latencies in the p.m. The stress-induced increase in the p.m. of brain met-enkephalin, furthermore, persisted in the hypophysectomized rats. This study suggest that the pituitary is not essential for the diurnal variation in responsivity to pain, the hyperalgesic activity of naloxone or the stress-induced increases in brain met-enkephalin.[206]

Time-dependent changes have also been detected in the concentration of beta-endorphin in the pituitary gland, the pons, the medulla, and the cerebellum of the rat brain. The studies on the stress-induced analgesia showed that peak levels of beta-endorphin peptide occurred during the activity period of the animals using the tail-flick and the hot plate tests in mice kept on a 12:12 L:D cycle and found that pain sensitivity was lower between 08.00 hr and 14.00 hr and significantly higher between 22.00 hr and 04.00 hr. The time of peak and low pain sensitivity was slightly different when the animals were kept on constant light, as the lowest and highest pain sensitivities were found between 22.00 hr and 02.00 hr and 0800 hr-22.00 hr, respectively. These results suggest that the rhythm of stress-induced analgesia was due to the release of an opioid peptide, since naloxone (5 mg/kg) reduced significantly the tail-flick test.[205,207,208] One study used a radioimmunological assay and quantified the opioid activity in humans and in

monkeys. They sampled human plasma and monkey cerebrospinal fluid at 2-hr intervals over a 24-hr period and they found an episodic secretion of an opioid substance with a morning peak and an evening dip. There was a 40% difference between peak and trough values of human plasma.[209]

Another study investigated the diurnal variation of opiate receptor binding. The amount of 3H-naloxone was measured every 4 hr across a 24-hr period in the forebrain of rats. These animals had been housed under a 12:12 L:D for 3 weeks. A significant circadian variation was found with a peak at 22.00 hr and a trough at 02.00 hr. The data indicate that the difference of binding throughout the day was due to changes in the number of binding sites rather than in the affinity of the drug for the receptor site.[210]

2.15. Diurnal variations in opioid requirement

The variations in the pain intensity can be derived from the opioid requirement. Opioid requirement not only varies diurnally but is also dependent on the type or cause of the pain. The influence of diurnal variation on the morphine requirements of patients using patient-controlled analgesia was studied. Forty-six patients undergoing either elective or gastric bypass surgery composed the study group. Patients were allowed to use the patient controlled analgesia machine for 36 to 72 hours postoperatively to deliver 0.6 mg/sq m doses of morphine sulfate intravenously. This study reveals that on postoperative days 1 and 2, a peak morphine requirement occurred at about 09:00h, whereas the lowest requirement was observed at 03:00h. This study suggests that there is a circadian variation in

narcotic analgesic need in the postoperative patient that is met appropriately by patient controlled analgesia.[200]

In a double-blind study, 55 patients who had undergone chest or stomach surgery were selected and received either continuous morphine infusions or four-hourly subcutaneous injections. Pain was assessed by using visual analogue scale and found that circadian variations in morphine administration were independent of the type of administration. The acrophase of pain occurred between 18:30 and 19:15h.[211]

In another study involving 19 female patients who had undergone surgery for gynecological carcinoma, where the patients self-administered either morphine sulphate or hydromorphone for pain relief using a pump at four-hourly intervals. The requirement for both opioids was significantly higher between 4.00 and 8.00 hr and significantly lowers for morphine between 12:00 and 16:00h and for hydromorphone between midnight and 4.00 hr. Morning doses were about 60% higher than doses administered at other times of day.[212]

In a further study, eight carcinoma patients were given a basal injection of hydromorphone, combined with patient controlled analgesia or with continuous infusion of the opioid. The pain intensities measured at 4-hour intervals using a visual analogue scale indicated that the pain was twice as severe at 22:00h as it was at 14:00h. Hydromorphone requirement peaked between 18.00 and 22:00h and was at its lowest between 2:00 and 6:00h.[213]

The time at which an analgesic is administered also appears to impact on the duration of analgesia. This was observed in a study performed in 77 healthy women in the active phase of labour, when they received subarachnoid fentanyl either during the day or night to relieve the pain of their contractions. Measurements of pain intensity on a visual analogue scale showed that analgesia persisted for longer after daytime administration than after nocturnal administration of the analgesic.[214]

2.16. Circadian rhythm and insulin secretion

2.16.1. In animals

It has been reported that plasma insulin concentration is dependant of circadian rhythm in several mammalian species, including humans, dogs, syrian hamsters, rats and mice.[26, 215-217] The circadian rhythm of insulin secretion from isolated rat pancreatic islets in vitro suggest that an endogenous circadian oscillator is located within the pancreatic islets of the rat that regulates circadian insulin secretion of the insulin-producing beta cells.[217]

Studies are supporting the diurnal variations in insulin secretion from the pancreas. It is reported that an endogenous circadian oscillator is located in the pancreatic islet of rat that regulates circadian insulin secretion - producing beta cells. A study was conducted on circadian rhythms in blood glucose and the effect of different lighting schedules, hypophysectomy, adrenal medullectomy and starvation in rats. The results showed that plasma concentrations of insulin and glucose are higher during dark cycle as compared to light cycle.[218]

Plasma insulin and glucose levels have been determined in normal rats, at different times during a 24-hour period. Plasma insulin levels show higher values during daytime, with a maximum at 5 p.m.[219] Another study on the night and daytime basal plasma insulin levels and insulin responses to an intravenous glucose load, basal blood glucose levels and rates of glucose uptake, and, finally, the diurnal patterns of blood glucose levels were investigated in normal compared to vagotomized Wistar rats. The study results showed that insulin secretion is greater in response to glucose load at night rather than during the day.[220]

Another important study was attempted to characterize the daily rhythm of plasma glucose, insulin in rats and to determine whether hormone rhythms occur independently of glucose variations. In that study, plasma glucose, insulin was investigated in rats infused for 24 h with a saline or glucose solution. The results of this study suggest the presence of insulin secretion rhythmicity independent of glucose variations and the existence of a diurnal plasma glucose rhythm with an increase occurring at the beginning of the night in fasted rats.[221]

The effects of glucose on insulin secretion and 86Rb efflux from isolated rat islets were studied at six different times during a 24-h period. The results showed that insulin secretion was not changed with the time of day in the presence of substimulatory concentrations and in the absence of glucose. The results showed that the time of day appears to affect insulin secretion mainly at glucose concentrations close to physiological values.[222]

2.16.2. In humans

Diurnal pattern of serum insulin and blood glucose is reported in a study conducted in the normal subjects.[223] Circadian variations in concentrations of plasma glucose, serum insulin, and C-peptide were assessed in 30 subjects during a 72-hour fasting period to evaluate rhythms independent from glucose supply. The finding suggests a central nervous system contribution to the regulation of insulin secretion independent of plasma glucose levels.[224]

Intravenous glucose tolerance tests were performed in the morning and afternoon on normal persons. The rate of decline of blood sugar was found to be all higher in the morning tests, and the mean values were significantly higher in the morning. Fasting blood sugar levels were slightly lower in the afternoon. There was no difference between the fasting morning and afternoon plasma insulin levels, but the levels after glucose were lower in the afternoon. Growth hormone levels were low at all times in non-apprehensive subjects and unaffected by glucose. The results suggest that the impaired afternoon intravenous glucose tolerance is associated with impaired insulin release and insulin.[225]

Circadian variations in blood glucose have been reported in healthy humans as well as in mammalian species and greater insulin secretion in response to a glucose overload is observed at night rather than during the day.[226, 221] Systematic diurnal variations were revealed for insulin secretion in humans.[227,228] Circadian rhythms of insulin secretion were observed in fasted or fed rats as well as in rats and humans subjected to hyperglycemic clamp. In addition, an endogenous control of the

circadian rhythm of insulinemia may be present as demonstrated in different animal species subjected to starvation including humans, rats and rabbits.[222]

Insulin secretion was studied in healthy volunteers at three different levels of glycemia. Plasma glucose was clamped at approximately 5, approximately 8.8 and approximately 12.6 mM for 68 h. Measured were serum insulin concentration. Rhythmic patterns of ISR were identified at all three glucose concentrations test. Serum insulin concentration changed in a circadian rhythm, increasing from a nadir between midnight and 6 a.m and reaching a peak between noon and 6 p.m. This study concluded that normal human subjects have a circadian rhythm of insulin secretion.[26]

The circadian and ultradian variations of blood glucose and plasma insulin have been characterized individually and as a group phenomenon in five healthy young adults studied. The subjects were studied in the laboratory and their life routine was controlled, but very close to that of their habitual routine. They had mainly ultradian rhythms of blood glucose and circadian rhythms of immunoreactive insulin. Blood glucose ultradian rhythms seem to be mainly but not exclusively meal time dependent, while immunoreactive insulin circadian rhythms appear to be primarily endogenous in origin. Therefore, the role played by insulin in the control of blood glucose levels seems to be programmed on a circadian basis rather than by a time independent feedback phenomenon as postulated by the conventional homeostatic hypothesis.[24]

2.17. Pentazocine

Pentazocine Lactate, a benzomorphan derivative is an opioid analgesic has been widely used for pain management. It is used for the relief of moderate to severe pain. It has both opioid agonist and antagonistic action. Agonistic action is predominantly κ-opioid receptor mediated and weak antagonistic action is μ-opioid receptor mediated.[229,230] Animal studies show that both mu opioid receptors and kappa opioid receptors can contribute to antinociceptive effects to somatic as well as visceral pain induced by pentazocine.[231,232] Intraperitoneal administration of pentazocine in mice at a dose of 30 mg/kg produces significant antinociceptive effect in formalin, tail flick and hot plate models.[233] Acetic acid abdominal constrictions were reduced by pentazocine in dose dependent manner.[234]

The analgesia produced by combinations of low-dose naloxone with pentazocine or morphine has been documented in humans and animals. The analgesia produced by pentazocine is potentiated by low-dose naloxone in both humans and animals. These data demonstrate a novel interaction between opiates and suggest a rationale for opiate combinations to produce potent analgesia with fewer autonomic side effects and less abuse potential than presently available analgesics.[21]

2.18. Methods for evaluation of pain in animal model:

There are various screening models available for inducing pain which includes centrally acting methods, peripherally acting methods. In centrally acting methods, thermal methods, chemical methods and mechanical methods are included.

2.18.1. Acetic acid induced method

This method is most commonly used methods for measuring peripheral analgesic activity. Pain is induced by injection of 10 ml/kg of acetic acid (0.6% in saline solution) into the peritoneal cavity of mice. The mice are placed individually in observation chambers immediately after the acetic acid injection, the number of abdominal constrictions produced by each mouse is counted for a period of 30 min.[5,13] In this test both central and peripheral analgesics are detected. Because of the lack of sensitivity, caution is required in interpreting the results. Nevertheless, a good relationship exists between potencies of analgesics in writhing assays and their clinical potenticies.[235] Acetic acid abdominal constrictions assay is regarded as very sensitive method employing minimal noxious stimulus and even weaker analgesics can be detected from the results of the test.[236]

2.18.2. Formalin Induced Method

Fifty microlitre of 1% formalin in saline was administered subcutaneously into the plantar surface of the left hind paw. The summation of time in seconds spent in licking and biting as an index of painful response which was determined at 15–30 min (late phase).[237] This method measures the ability of drug to attenuate moderate continuous pain generated by tissue.[238] The acute phase represent neurogenic pain behavior and the chronic phase represents inflammatory pain behavior.[229]

2.18.3. Tail Flick Method

The analgesic activity in mice was determined by radiant heat tail-flick method. Tail-flick latency was assessed by the analgesiometer (Inco, India).The strength of the current passing through the naked nichrome wire was kept constant at

5A. The distance between heat source and the tail was 1.5 cm and the application site of the heat on the tail was within 2 cm, measured from the root of the tail. Tail-flick latency was measured after 30 min from the administration of drug.[239] This method is very effective to estimate the efficacy and potency of central acting analgesic drugs.

2.18.4. Hot plate method

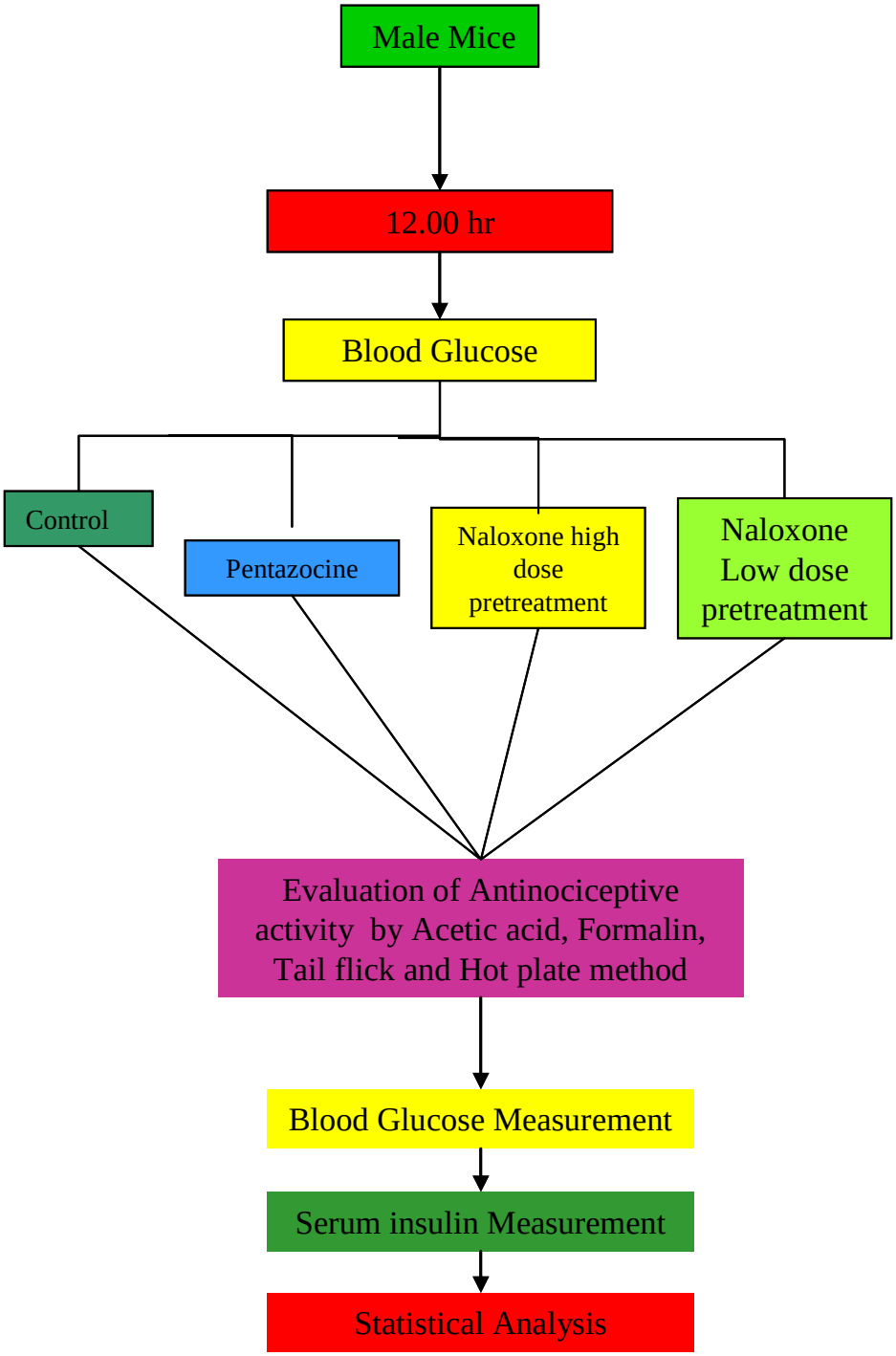
The hot plate test is commonly used for measuring central analgesic activity. The hot plate was maintained at 55±0.5 °C. Animals are placed on the hot plate and the time between placement and licking of the hind paws or jumping was recorded as the index of response latency. A cut-off time of 30s is maintained to minimize tissue damage.[240] The paws of mice are very sensitive to heat at temperatures which are not damaging the skin. The responses are jumping and licking of the paws. The time until these responses occur is prolonged after administration of centrally acting analgesics.[229.] A study was conducted to assess the effects of dimecron on the endocrine response in blood by measuring serum levels of different hormones. Serum insulin was measured by using insulin ELISA kit (Cal Biotech Inc., Spring Valley, USA).[241]

The above mentioned review of literature reveals the association between endogenous insulin and antinociception independent of the glycemic status and also the involvement of opioids in such an association. It is also reported that endogenous insulin is influenced by diurnal rhythm. However, to our knowledge, only few reports are available on the role of endogenous insulin in pain threshold and no report is available in the literature on the effect of diurnal rhythm on the relation

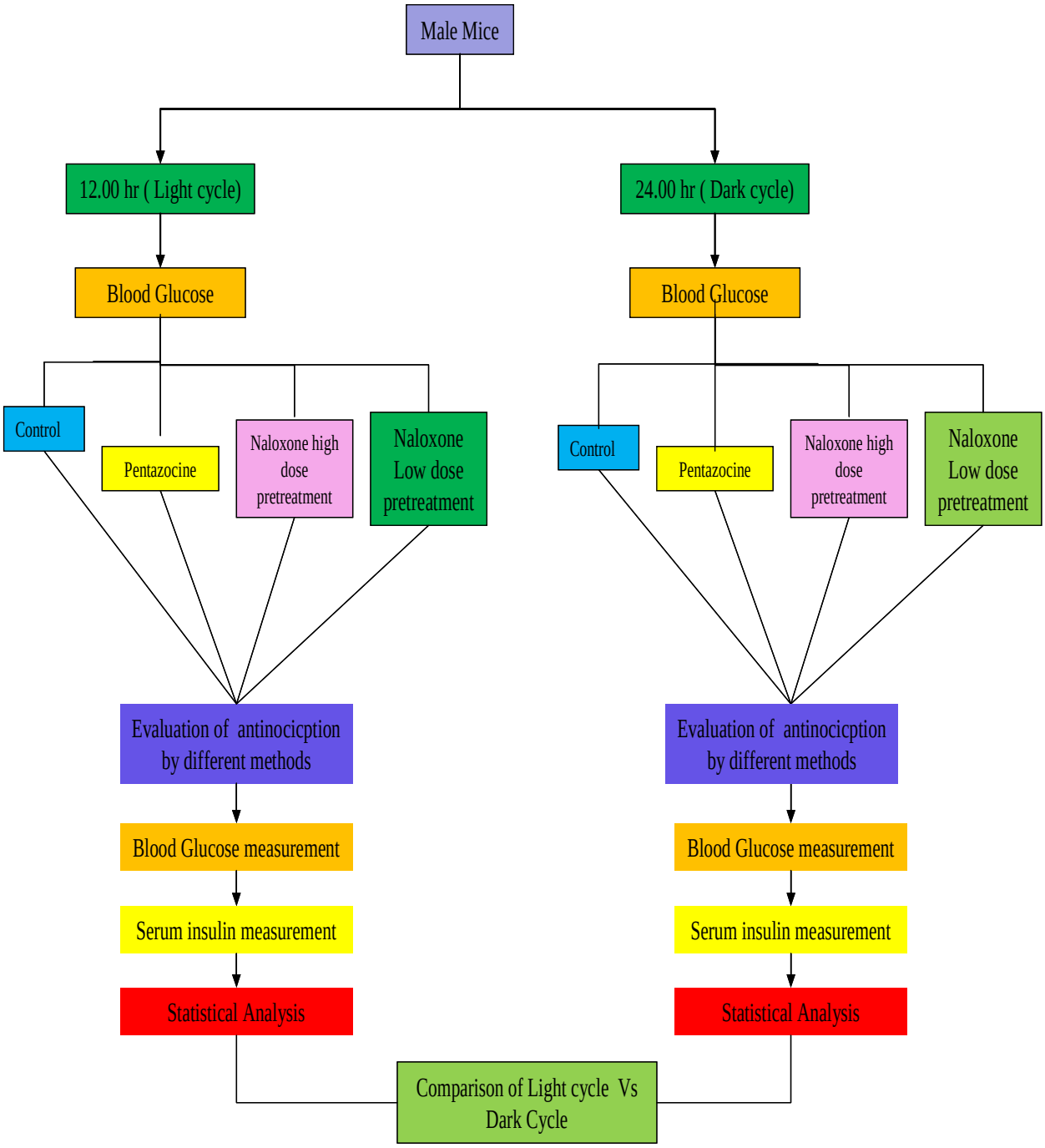
between endogenous insulin and antinociception in different pain models. So, the present study was directed to investigate the role of endogenous insulin in pain threshold and the relation between endogenous insulin and antinoiceception using pentazocine, the opioid analgesic, as a model drug in mice. The results of the study may help to understand new insight into the insulin pathway if any that influences antinociception induced by opioid analgesics and also how it is influenced by diurnal rhythm.

PLAN OF WORK

1. FLOW CHART OF EXPERIMENTAL WORK



2. FLOW CHART OF EXPERIMENTAL WORK



5. METHODOLOGY

5.1. Animals

For the experimental study, randomly bred healthy adult male Swiss albino mice (25 to 30 gm) were used. They were housed in polypropylene cages in groups of 6 with free access to food (pellets obtained from Gold Mohar Ltd., Bangaluru, India) and water. They were maintained under normal room temperature ranging between 28-30^{o} C with 12:12 h light:dark cycle for a period of one week prior to experimentation. All the animals were acclimatized for three days (1 h/ day) to the experimental setting, with white light on for experiment in light cycle (12.00 h) and red light bulb on for experiment in dark cycle (24.00 h) by placing in a cage with a stainless steel grid and dark opaque plastic walls. The experiments on animals were performed after the approval of the protocol by the Institutional Animal Ethical Committee. (SVCP/ IAEC/ Ph.D/ 01/2011)

5.2. Drugs and chemicals

Pentazocine lactate (Ranbaxy Pharma, India), Naloxone hydrochloride (Endo Labs, USA), Glacial acetic acid (Loba Chemie, India), Formalin (Spectrum Reagents and Chemicals Pvt. Ltd., Cochin, India), Insulin ELISA kit (Cal Biotech Inc., Spring Valley, USA).

5.3. Assessment of nociception and antinocieption

The nociception and antinociception effects were assessed by different nociceptive tests such as Acetic acid induced abdominal constrictions method,

Formalin induced method, Tail flick method and Hot plate method at 12.00 h for light cycle and 24.00 h for dark cycle.

5.3.1. Acetic acid induced abdominal constrictions method

Animals received 10 ml/kg of 0.6% freshly prepared acetic acid intraperitoneally. The number of abdominal constrictions 15 min following this injection was noticed and considered as nociceptive response. A significant reduction in number of abdominal constrictions ($P < 0.05$ level) was considered as an antinociceptive response. Every animal was exposed to this test only once to avoid the fluctuation in the responses caused possibly due to the neural damage by acetic acid. A minimum number of 6 animals were included in each group.[236]

5.3.2. Formalin Induced Method

50 microlitre of 1% formalin in saline was administered s.c into the plantar surface of the left hind paw. The time in seconds spent in licking and biting the injected paw was recorded.. The early phase (acute pain) of nociceptive response normally peaks from 0 to 10 min and the late phase (chronic pain) from 10 to 30 min after formalin injection. In the present study, we measured late phase only. The time in seconds spent in licking and biting the formalin injected paw from 10 to 30 minutes and considered as the quantitative indication of nociception. A significant reduction in response time from 10 to 30 min period ($P < 0.05$ level) was considered as an antinociceptive response. Every animal was exposed to this test only once to avoid the fluctuation in the responses. A minimum number of 6 animals were included in each group.[237, 238]

5.3.3. Tail flick Method

Portion of the tail of animals were subjected to radiant heat using analgesiometer (Inco, India). The strength of the current passing through the naked nichrome wire was kept constant at 5A. The distance between heat source and the tail was 1.5 cm and the application site of the heat on the tail was within 2 cm, measured from the root of the tail. The tail flick latency time in sec was noticed and considered as the quantitative indication of nociception. A significant increase in tail flick latency time in seconds from 10 to 30 min period ($P < 0.05$ level) by any treatment compared with control was considered as an antinociceptive response. Every animal was exposed to this test only once to avoid the fluctuation in the responses. A minimum number of 6 animals were included in each group.[239]

5.3.4. The Hot Plate Method

Animals were placed on the hot plate maintained at 55±0.5°C and the time between placement and licking of the hind paws or jumping was recorded as the index of response latency and considered as nociceptive response. A cut-off time of 30 sec was maintained to minimize tissue damage. Every animal was exposed to this test only once. A minimum number of 6 animals were included in each group.[240]

5.4. Role of Opioid System

The role of opioid system in the possible changes on endogenous insulin level in pain threshold was investigated by pretreating the animals with naloxone (5mg/kg; i.p.) and after 15 min the animals were treated with pentazocine. The antinociceptive response, the blood glucose and serum insulin level were recorded

prior to the exposure of any drug/nociception to the animal, after the drug treatment; just prior to nociceptive challenge and at the end of nociceptive challenge.

5.5. Assessment of Diurnal rhythm on Endogenous insulin, Blood glucose level and Pain threshold

The possible influence of diurnal rhythm on endogenous insulin, blood glucose level and pain threshold in dark cycle (24.00 h) and in light cycle (12.00 h) during nociception and antinocicpetive effects of pentazocine and low dose naloxone was studied by using the different nociceptive methods described earlier. The role of opioid system on the parameters was studied by using naloxone (5 mg/kg i.p.). The assessment of nociception, antinociception, role of opioid system, measurement of blood glucose and serum insulin during dark and light cycle were done in mice as described earlier.

5.6. Treatments

Animals received the following treatments

- Vehicle control (saline, 10 ml/kg i.p.) 30 min prior to nociceptive challenge.
- Pentazocine treatment (4mg/kg i.p. in acetic acid induced abdominal constrictions methods: 30mg/kg i.p. in formalin induced method, Tail flick method and hot plate method) 30 min prior to each nociceptive challenge.
- Naloxone low dose pretreatment (0.005mg/kg, i.p.) 15 min prior to pentazocine treatment in each pain model.

- Naloxone high dose pretreatment (5mg/kg, i.p.) 15 min prior to pentazocine treatment in each pain model.

5.7. Measurement of Blood Glucose

Blood was collected from animal by cutting the tip of its tail before and after respective treatment at the appropriate time and estimated for glucose by allowing it to react with appropriate glucostrip.[242]

5.8. Measurement of Serum Insulin

Blood was collected from animal by tail vein method before and after respective treatment at the appropriate time. It was allowed to stand for 10 min and serum was collected from the blood by centrifuging the tubes at 3000 rpm in centrifuge (REMI, Mumbai, India). The serum was separated in serum tubes and it was stored at appropriate condition. The estimation of serum insulin was carried out using insulin ELISA kit (Cal Biotech Inc., Spring Valley, USA). The following procedure was adopted for the measurement of serum insulin by ELISA method.

- Securet the desired number of coated wells in the holder and marked the date sheet with sample identification.
- Dispensed 25 µl of serum sample, control and reference into the assigned wells.
- Dispensed 100 µl of Enzyme conjugate into each well and mix for 10 seconds.
- Incubated for 60 minutes at room temperature.

- Removed liquid from all wells and washed three times with 300µl of 1X wash buffer blot on absorbent paper.
- Dispensed 100 µl of TMB Solution into each well.
- Incubated for 15 minutes at room temperature.
- Stop reaction by adding 50 µl Stop Solution to each well and read absorbance at 450 nm with a micro well reader. Standard graph was plotted with the help of absorbance values against concentrations of known insulin levels and the unknown was measured by interpolating the absorbance values in the graph.

5.9. Interaction between insulin and opioid receptors

We studied the possible interaction between insulin and opioid receptors by protein - protein docking method using clusPro 2.0 program. The results are shown in Table 9 and 10, in Figure 30 and 31. For this preliminary study, protein structures for human insulin protein (PDB code : 4DJH) and human kappa receptor (PDB code : 4DJH) were used for studying interaction between them using cluspro 2.0 Program (Figure 30 and 31), (Table 9 and 10).

5.10. Statistical Analysis

The data were subjected to statistical analysis by using one way ANOVA followed by Dunnett's't' test. A significant level of $P < 0.05$ was considered for responses. Student's' test was used for comparison of diurnal rhythm effects between two groups at 12.00 h and at 24.00 h.

6. RESULTS

6.1. Relation between serum insulin, pain threshold and blood glucose with various treatments against different nociceptive tests.

6.1.1. In acetic acid induced abdominal constrictions method

The effects of pentazocine (4mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p) on serum insulin, pain threshold and blood glucose in acetic acid induced abdominal constrictions method are reported in Table 1 and Fig. 6, 7 and 8. Pentazocine treatment antagonized the abdominal constrictions significantly ($P<0.01$) with significant increase in serum insulin level ($P<0.01$) without any change in blood glucose level. Naloxone (high dose) pretreatment in pentazocine treated animals did not significantly influence the blood glucose level, however, antagonized the pentazocine induced antinociception with decrease in serum insulin level significantly. Low dose naloxone pretreatment in pentazocine treated animals significantly ($P<0.01$) reduced the abdominal constrictions with elevated serum insulin level ($P<0.01$) with no change in blood glucose level.

6.1.2. In formalin induced nociceptive method

The effects of pentazocine (30mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p) on serum insulin, pain threshold and blood glucose in formalin induced nociceptive method are shown in Table 2 and Fig. 9, 10 and 11. Pentazocine treatment reduced the response time significantly ($P<0.01$) with no significant change in blood glucose level as compared to control; however, serum

insulin level was significantly (P<0.01) increased. Naloxone (high dose) pretreatment in pentazocine treated group did not significantly change blood glucose level; however the serum insulin level and the response time was significantly (P<0.01) reduced. **N**aloxone (low dose) pretreatment in pentazocine treated animals significantly (P<0.01) increased the serum insulin level with significant reduction (P<0.01) in response time as compared to pentazocine treatment, however, the blood glucose level was not changed.

6.1.3. In tail flick method

The effects of pentazocine (30mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p) on serum insulin, pain threshold and blood glucosein tail flick method are illustrated in Table 3 and Fig. 12, 13 and 14. Pentazocine treatment significantly reduced tail flick latency time (P < 0.01) with no change in blood glucose level; however, serum insulin level was significantly (P < 0.01) increased. Naloxone (high dose) pretreatment in pentazocine treated group did not significantly change the blood glucose level as compared to control; however showed significant decrease in serum insulin level as well as tail flick latency time. Naloxone (low dose) pretreatment in pentazocine treated animals significantly (P < 0.01) increased the serum insulin level with significant reduction in latency timeas compared to pentazocine treatment; however, the blood glucose level was not changed significantly.

6.1.4. In hot plate method

The effects of pentazocine (30mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p) on serum insulin, pain threshold and blood

glucosein hot plate method are given in Table 4 and Fig. 15, 16 and 17. Pentazocine treatment significantly **(**P<0.01**)** reduced the blood glucose level with increase in serum insulin level **(**P<0.01**)** as well as latency time **(**P<0.01**)**. Naloxone (high dose) pretreatment increased blood glucose level significantly (P < 0.01) in pentazocine treated animals with decrease in serum insulin level in latency time as compared to pentazocine treatment. Naloxone (low dose) pretreatment in pentazocine treated animals significantly (P < 0.01) increased the serum insulin level with significant reduction in latency time **(**P<0.01**)** as compared to pentazocine treatment; however, the blood glucose level was not changed significantly.

6.2 Effect of diurnal rhythm on pain threshold, endogenous insulin and blood glucose with various treatments against different nociceptive tests.

6.2.1. Effect of diurnal rhythm on pain threshold, endogenous insulin and blood glucose in control, pentazocine, naloxone high dose and naloxone low dose in acetic acid induced abdominal constrictions method

The effect of diurnal rhythm on pain threshold, endogenous insulin and blood glucose in control, pentazocine (4mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p) in acetic acid induced abdominal constrictions method are shown in Table 5 and Fig. 18,19 and 20. In acetic acid induced abdominal constrictions method, as compared to light cycle, number of abdominal constrictions in the dark cycle was found to be significantly lower (P<0.0001). In other words, the pain threshold was higher in the dark cycle than in the light cycle. Pentazocine treatment significantly reduced the number of abdominal constrictions in both light and dark cycle. As compared to number of abdominal

constrictions in the control, the effect of pentazocine was found to be greater ($P<0.0001$) in the dark cycle than in the light cycle. Pentazocine treatment significantly reduced the abdominal constrictions about 5 folds in the dark cycle as compared to 2 fold in the light cycle.

Naloxone (high dose) pretreatment reversed the pentazocine induced antinociception to values close to that of control values for abdominal constrictions in dark as well as light cycle. Serum insulin level was found to be significantly higher in the dark cycle ($P<0.007$) than in the light cycle in the control animals. Naloxone (high dose) significantly reduced the serum insulin level in pentazocine treated animals in both dark and light cycle. The serum insulin level was reversed by naloxone pretreatment to values close to that of control in both dark and light cycle. Significant differences in serum insulin level in control ($P<0.007$), pentazocine treatment ($P<0.02$) and naloxone (high dose) pretreatment ($P<0.002$) were observed between dark and light cycle. Serum insulin level was found to be higher in the above treatment in the dark cycle as compared to light cycle. The changes in serum insulin level were found to be independent of the glycemic status (Table 5).

Naloxone (low dose) pretreatment potentiated the pentazocine induced antinociception by decreasing the abdominal constrictions significantly ($P < 0.01$) in the dark cycle. Similarly in the light cycle, naloxone (low dose) pretreatment reduced the abdominal constrictions in the pentazocine treated group significantly ($P<0.01$) with corresponding increase in the serum insulin level in both dark and light cycle. A significant difference in number of abdominal constrictions ($P < 0.0001$) and in serum insulin ($P < 0.002$) was observed between light and dark cycle.

Naloxone (low dose) pretreatment did not significantly influence the blood glucose level in the pentazocine treated animals in both dark and light cycle (Table 5).

6.2.2. Effect of diurnal rhythm on pain threshold, endogenous insulin and blood glucose in control, pentazocine, naloxone high dose and naloxone low dose in formalin induced method.

The results of the effect of diurnal rhythm on pain threshold, endogenous insulin and blood glucose in control, pentazocine (30mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p) informalin induced method are illustrated in Table 6 and Fig. 21, 22 and 23. In formalin induced method, the response time was found to be less in the dark cycle than that in the light cycle ($P<0.0001$). In pentazocine treated animals, the serum insulin level was higher ($P<0.009$) in the dark cycle than that observed in the light cycle. No significant change in blood glucose level was observed either in the dark cycle or in the light cycle and also there was no significant difference in blood glucose level between dark and light cycle. Pentazocine treatment in formalin induced nociception in mice significantly reduced the response time in the dark cycle as well as light cycle and the percent inhibition of response time was higher in the dark cycle than in the light cycle. Naloxone (high dose) pretreatment reversed the pentazocine induced response time as well as the serum insulin level to the values close to that of control. The serum insulin level was comparatively higher in the dark cycle than in light cycle ($P<0.01$) following naloxone (high dose) pretreatment in the pentazocine treated animals. Naloxone (high dose) pretreatment did not significantly influence the blood glucose level in both in the dark and light cycle but showed significant difference in

response time (P < 0.0001) and in serum insulin (P < 0.0001) with no influence on blood glucose level in both in the dark and light cycle (Table 6).

There was a mild potentiation of pentazocine induced response time by naloxone (low dose) pretreatment with significant increase in serum insulin level in both dark and light cycle. The effect of naloxone (low dose) on pentazocine induced response time was greater in the dark cycle (P<0.0001) than that observed in the light cycle. Similar pattern of changes in serum insulin level was observed in the dark as well as light cycle (P<0.01). No significant change in the blood glucose level was observed in both dark and light cycle by naloxone (low dose) (Table 6).

6.2.3. Effect of diurnal rhythm on pain threshold, endogenous insulin and blood glucose in control, pentazocine, naloxone high dose and naloxone low dose in tail flick model.

The effect of diurnal rhythm on pain threshold, endogenous insulin and blood glucose in control, pentazocine (30mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p) in tail flick model are reported in Table 7 and Fig. 24, 25 and 26.In tail flick model, control animal showed increase in latency time in the dark cycle as compared to that in light cycle (P<0.001). The serum insulin level was also found to be higher in dark cycle as compared to that in light cycle in the control animals (P<0.0001). Pentazocine pretreatment increased the latency time significantly with significant increase in the serum insulin level in the dark as well as light cycle. Significant difference in the latency time (P<0.001) as well as serum insulin level in pentazocine treated animals (P<0.03) was observed between dark and light cycle. Naloxone (high dose) significantly reduced the latency

time as well as serum insulin level in the pentazocine treated animals in the dark as well as light cycle. There was difference in the insulin ($P<0.03$) level as well as in the latency time ($P<0.04$) between dark and light cycle in these animals. Naloxone (high dose) did not significantly influence blood glucose level in both dark and light cycle (Table 7).

Naloxone (low dose) potentiated the pentazocine induced latency time with corresponding increase in serum insulin level both in the dark cycle and in the light cycle. The potentiation by naloxone (low dose) was found to be significantly higher in the dark cycle as compared to that in the light cycle ($P<0.0001$). Similarly, significant difference in serum insulin level ($P<0.0008$) was observed between dark and light cycle in the naloxone (low dose) treated animals. There was no significant change in the blood glucose level in pentazocine treatment or in the naloxone (low dose) treatment in both dark cycle and light cycle (Table 7).

6.2.4. Effect of diurnal rhythm on pain threshold, endogenous insulin and blood glucose in control, pentazocine, naloxone high dose and naloxone low dose in hot plate method.

The results of the effect of diurnal rhythm on pain threshold, endogenous insulin and blood glucose in control, pentazocine (30mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p) in hot plate method are shown in Table 8 and Fig. 27, 28 and 29. In the hot plate method, pentazocine treatment significantly ($P<0.01$) increased the latency time with corresponding increase in serum insulin level as compared to control, however, there was decrease in the blood glucose level in the pentazocine treated animals. Comparision between

dark and light cycle on the effect of pentazocine treatment shows the magnitude of increase in latency time was significantly greater ($P<0.0001$) in the dark cycle as compared to light cycle. While significant difference in the latency time as well as serum insulin level ($P<0.01$) was observed between dark and light cycle, no significant difference in the blood glucose level was observed between dark and light cycle with pentazocine treatment. Naloxone (high dose) reversed the changes in the latency time, serum insulin and blood glucose level to control values. However, a significant difference was observed between dark and light cycle in latency time ($P<0.0001$), serum insulin level ($P<0.002$) in control, pentazocine treated and naloxone (high dose) pretreated mice (Table 8).

Naloxone (low dose) potentiated the pentazocine induced latency time with corresponding increase in serum insulin level both in the dark cycle and in the light cycle. The potentiation by naloxone (low dose) was found to be significantly higher in the dark cycle as compared to that in the light cycle ($P<0.0001$). Similarly, significant difference in serum insulin level ($P<0.01$) was observed between dark and light cycle in the naloxone (low dose) treated animals. There was no significant change in the blood glucose level in the naloxone (low dose) treatment as compared to pentazocine treatment in both dark cycle and light cycle (Table 8).

6.3. Protein-protein docking study between kappa receptor and insulin using clusPro 2.0 program.

The results of the preliminary docking study between kappa receptor and insulin are shown in Table 9, 10 and Fig. 30 and 31. The number of hydrogen bonds between A and B chain were 12. The number of interface residues between A and

Chain were 31: 24. As it is clear from our study results the involvement of insulin in the modulation of pain mediated through opioid pathway, we analyzed the interaction if any, between insulin and opioid receptors by protein-protein docking method and we observed insulin binding with opioid receptors in particular kappa receptor through hydrogen bonding; however, the active site of the opioid receptor that binds with the aminoacids residue of insulin could not be clearly established.

7. DISCUSSION

The present study demonstrates the involvement of endogenous insulin in pain threshold through opioid pathway which is independent of the glycemic status. Furthermore, the association between endogenous insulin and pain threshold seems influenced by the type of nociceptive stimuli and also by diurnal rhythm.The results of diurnal rhythm obtained from the experiments in the animal model cannot be extrapolated to humans considering the differences in diurnal rhythm between humans and animals. This study was limited to the measurement of endogenous insulin rather than c-peptide level in response to nociceptive stimuli as well as pentazocine treatment.

7.1 Association between endogenous insulin and pain threshold and its interaction with nociceptive stimuli

Pain is a complex phenomenon and several chemical mediators, neurotransmitters and neuropeptides are involved in pain perception.[22] In the recent past, the involvement of insulin in the modulation of pain has assumed significance; however mechanisms underlying this are not clearly understood. Factors influencing insulin secretion from pancreas have been reported. Studies show that beta endorphin present in pancreas involves insulin release.[16] The beta-endorphin potentiates insulin secretion via a common beta cell opioid receptor, and that beta-endorphin may exert a paracrine control of insulin secretion.[19] Opioid analgesics mimic endogenous opioid peptides and therefore have influence on insulin release from pancreatic islets.[17] Furthemore, opioid receptors are present in the pancreas[18]

and insulin is elevated by opioids by the action at the islet cells.[63] Insulin secretion is also mediated through mu opioid receptor activation and opening of K_{ATP} channels.[62]

Morphine stimulates the insulin and glucagon release from the perfused dog pancreas and in high concentrations increases the circulating levels of insulin without change in glucose.[16] The involvement of exogenous insulin in the modulation of pain has also been reported by few studies. Insulin potentiates the antinociceptive response of sodium salicylate[7] and morphine[8] and i.c.v. injection of insulin dose dependently lower the second phase of formalin induced pain but it does not significantly lower the blood sugar level and suggest participation of opioid mechanism in insulin on pain threshold.[5] Clinical evidence shows that normal subjects with higher insulin level have elevated threshold for thermal nociceptive stimuli.[12] However study is scarce as to the role of endogenous insulin in pain threshold except in one study in animal model which claims association between endogenous insulin and pain threshold which is independent of the glycemic status.[13] Most previous studies followed nociceptive test either in formalin nociception or acetic acid induced nociception on studying the association between insulin and pain threshold besides no established opioid analgesic was involved in the study.

In order to estimate the level of endogenous insulin during painful condition using behavioural nociceptive tests, it is essential to employ different tests which differ in stimulus quality, intensity and duration[238]. Acetic acid induced assay is regarded as a very sensitive method employing minimal noxious stimulus.[236]

Formalin induced nociception represents neurogenic (early phase) and inflammatory (late phase) pain behavior.[238, 237] Hot plate assay employs a high degree of thermal nociception.[240]

As observed in the literature, no detailed study was available on the association between endogenous insulin and pain threshold and how it is influenced by different nociceptive stimuli which formed the objective of the present study. The study was carried out using pentazocine, an opioid analgesic, as the model drug which is most commonly used in clinical practice.

The intensity of pain varies with the type of nociceptive stimuli employed and as such it becomes necessary to examine whether the intensity of pain has influence on the endogenous insulin and pain threshold and therefore in the present study, the different nociceptive tests such as formalin model, acetic acid model, tail flick model and hot plate model were employed to investigate the above said objective. We observed in the present study, no significant differences between nociceptive tests on the association between endogenous insulin and pain threshold; whenever the antinociceptive response to nociceptive stimuli changed, a significant change in serum insulin level was observed. Pentazocine treatment significantly reduced paw licking response time and the number of abdominal constrictions in formalin model and acetic acid model respectively. In tail flick as well as hot plate model, pentazocine treatment significantly increased the latency time. Irrespective of the nociceptive tests, as in formalin, acetic acid and tail flick models employed, we observed significant increase in serum insulin level without significant change in blood glucose level, whenever the antinociceptive response was observed with

pentazocine treatment. In other words, whenever there was increase in pain threshold due to the effect of opioid analgesics, there was significant increase in serum insulin level without affecting the blood glucose level. These findings clearly point to suggest that there is involvement of endogenous insulin in the pain threshold which is independent of the glycemic status and our finding is in agreement with previous reports.[5, 13]

While the formalin, acetic acid and tail flick models showed the involvement of serum insulin independent of the glycemic status in opioid antinociception, the hot plate model failed to support the above finding. There was significant increase in serum insulin level with increase in latency time in the antinociceptive response induced by pentazocine; however a significant change in blood glucose level was observed in the hot plate model. The tail flick model with low pain intensity showed association of endogenous insulin with pain threshold whereas the hot plate model with higher pain intensity failed though support the above said contention, but glucose dependently; possibly the intensity of pain seems to influence the glycemic status and as such the relation between serum insulin and pain threshold could not be clearly ascertained in the hot plate model. This finding tends to raise the question whether the involvement of endogenous insulin in pain threshold independent of the glycemic status is model dependent (pain intensity dependent).

The insulinemic and glycemic status before and after each nociceptive test was also examined. There was significant decrease in serum insulin level with significant increase in blood glucose level in response to nociceptive stimuli in all the pain models. Upon subjecting the animals to opioid treatment (pentazocine), we

observed significant increase in serum insulin without affecting the blood glucose level, whenever the antinociceptive response was elicited except in the hot plate model. This finding further supports the earlier contention that the association between serum insulin and pain threshold seems model dependent and the factor influencing is unknown.

7.2. Role of naloxone on endogenous insulin and pain threshold

The effect of naloxone in low as well as high dose on the involvement of endogenous insulin in pain threshold in all the four pain models was investigated. Naloxone in high dose normally antagonizes opioid mediated analgesic response. In contrast, low dose naloxone seems to have mild antinociceptive response which was evident in all the models investigated.[21] Naloxone pretreatment in low dose in pentazocine treated group significantly increased the serum insulin level without causing significant change in blood glucose level which correlated with significant decrease in response to nociceptive stimuli in all the four pain models employed in the study, thus establishing the potentiated analgesic response of pentazocine. The results further substantiate the involvement of endogenous insulin in the analgesic response whether induced by pentazocine or by low dose naloxone. The above finding further suggests that endogenous insulin is implicated in the modulation of pain through opioid pathway. Naloxone in high dose antagonized the pentazocine induced antinociception simultaneously reversing the elevated serum insulin level to control value (nociceptive stimuli value) without affecting blood glucose level thus evidencing the above said contention that there exists a relation between serum insulin and antinociceptive response that utilizes the opioid pathway. However, in a

study conducted on flavone (opioid analgesic) induced antinociception in mice in acetic acid model, it was reported that high dose naloxone administration though antagonized the flavone induced antinociception did not significantly influence the serum insulin level with changes in blood glucose level.[13] This observation proposes the view whether the involvement of endogenous insulin in the pain threshold is dependent on the type of opioid analgesic employed.

7.3 Association between endogenous insulin and pain threshold and its interaction with diurnal rhythm

Pain is a complex phenomenon and as such is still difficult and frustrating to treat even with newer devices such as slow release preparations or patient controlled analgesia pump; large inter-individual variation are found from one patient to the next both in pain intensity and in the doses of opioid self administered by the patients. Studies carried out in the last decade have also indicated time - dependent variations in the intensity and in the neurochemistry of pain as well as in the effects of analgesic drugs. Many studies report the circadian variation in the sensitivity of the pain.[22, 174-180]

Circadian rhythm influences the expression of pain and body's responsiveness to analgesic medication.[167,198] The suprachiasmatic nucleus is the primary pacemaker for the circadian physiologic systems and controls the circadian, ultradian and episodic release of beta endorphins.[157] Endorphins and enkephalins are endogenous peptides found in the region of brain known to be involved in perception of pain. Their brain concentrations are fluctuating over 24 hour's period.[187] Circadian variations in pain intensity in experimentally induced pain in

animals have also been reported, wherein a time- dependent change in pain intensity in the hot plate test was observed. [187]

Studies have also been conducted in clinical situations and circadian variations have been described in pain. The chest pain induced by myocardial infarction is more pronounced in the morning which is well known to clinicians as the rhythmic appearance of infarction. Circadian variations in pain intensity were reported in tooth ache, biliary colic, migraine, arthritis and bach ache.[174, 176, 178, 183] The above studies clearly evidence the fact the circadian variations are present in the pain intensity in humans as well as in animals. The existence of a diurnal plasma insulin rhythm independent of blood glucose variations has been reported.[221] There is greater insulin secretion in response to glucose load at night rather than during the day.[220, 225] However, the influence of diurnal rhythm in plasma insulin level and plasma glucose remains controversial. Some authors suggest increase in blood glucose during the day compared to night [219] whereas others report an increase in glucose level at night.[218]

Studies are supporting the diurnal variations in insulin secretion from the pancreas. It is reported that an endogenous circadian oscillator is located in the pancreatic islet of rat that regulates circadian insulin secretion - producing beta cells.[217] It has also been reported that plasma insulin concentration is dependant of circadian rhythm in several mammalian species, including humans, dogs, syrian hamsters, rats and mice.[26,215-216] Circadian rhythms of insulin secretion were observed in fasted or fed rats as well as in rats and humans subjected to hyperglycemic clamp. In addition, an endogenous control of the circadian rhythm of

insulinemia may be present as demonstrated in different animal species subjected to starvation including humans, rats and rabbits.[222] As evident from the previous studies, there has not been any attempt in studying the influence of diurnal rhythm on the relation between endogenous insulin and pain threshold and therefore the present study also included to examine this.

The pain intensity to nociceptive stimuli in all the pain models was found to be significantly lower in the dark cycle as compared to light cycle suggesting the diurnal variation in the pain intensity which is independent of the nociceptive tests employed. Our finding is thus in agreement with the previous reports.[163] Similarly endogenous insulin level was found significantly higher in the dark cycle as compared to light cycle and serum insulin was negatively correlating with the pain intensity, that is, as the insulin level decreased the pain intensity increased and vice versa in all the pain models tested in mice. These effects were found to be independent of the blood glucose level in all the models except in hot plate model. There was significant change in the blood glucose level in the hot plate model although serum insulin correlated with pain intensity as observed in other models. Thus it is evident that endogenous insulin associates with pain intensity which is influenced by diurnal rhythm. Though there were differences in pain intensity between dark and light cycle, the significance of blood glucose level changes in hot plate test on the relation between endogenous insulin and pain threshold is difficult to understand.

7.4. Role of diurnal rhythm on effects of naloxone

The influence of diurnal rhythm on the opioid mechanism was also examined using naloxone, the opioid antagonist. Naloxone in low dose as mild analgesic significantly reduced the pain intensity in the pentazocine treated animals with significant increase in serum insulin in both light and dark cycle; however differences in the pain intensity as well as serum insulin level were observed between dark and light cycle with no change in the glycemic status in all the pain models. High dose naloxone (opioid antagonist) reversed the elevated insulin level with pentazocine treatment to value close to control value without significant influence on the glycemic status except in hot plate model and differences in these effects were also observed between dark and light cycle. Therefore it is clear that the endogenous insulin associates with the pain threshold through opioid pathway which is influenced by diurnal rhythm.

7.5. Mechanisms underlying the association between endogenous insulin and pain threshold

Peptides such as endorphins and enkephalins are involved in antinociceptive action. Noxious stimuli that accompany physiological stressors are interpreted as unpleasant by most animals and humans.[46] To control the level of pain perceived, the body has developed mechanisms for inhibiting painful stimuli. Chief among these adaptive mechanisms is an inhibitory system based on production of beta endorphin. Beta endorphin is an endogenous opioid that is released in response to pain and increases the inhibition of pain at several sites. It is synthesized in various biological structures and released when animals are exposed to stress or painful stimuli.[44, 48-49.]

There is some evidence for the existence of opiates in the pancreas from immunofluorescent work.[51, 53, 67] Beta endorphins and enkephalins are present in the endocrine pancreas and therefore they are implicated in the neuroendocrine control islet hormone release.[32] Beta endorphins being opioid peptide influence the secretion of insulin in humans[14] and also in many animal species.[37- 39] It has also been reported that opioid receptors are present in human as well as animal pancreatic islets and further opioid analgesics mimics endogenous opioid peptides and therefore has influence on insulin release from pancreatic islets.[18, 17] Dynorphins a potent opioid peptide and kappa receptor agonist originally found in the pituitary and duodenum has also been found in the pancreatic is lets which also stimulates insulin release from the pancreas. [55, 58] The aforementioned reports indicate that opioid peptides influence the release of insulin from the pancreas.

Though previous study supports the involvement of endogenous insulin in pain threshold the underlying mechanism has not been clearly established.[13] This relationship can be ascribed to several influencing factors as reported in previous studies.[5] Insulin attenuates chronic rather than acute pains through a mechanism mediated by dopamine, 5- hydroxytryptamine and opioids.[5] It has been reported that morphine at an antinociceptive dose causes the release of endogenous opioid peptides and may also stimulate the biosynthesis of precursor molecules preproopiomelanocortin, preproenkephalin A and preproenkephalin B.[15]

In the present study, the role of opioid mechanism on the involvement of endogenous insulin in the modulation of pain was tested using high dose naloxone, an opioid antagonist. The observations that the pretreatment with high dose

naloxone reversed the elevated serum insulin level significantly to the control value (nociceptive stimuli value) in association with significant reduction in antinociceptive action of pentazocine independent of the glycemic status in acetic acid, formalin and tail flick pain models tend to suggest the role of opioid mechanism on the involvement of endogenous insulin in the modulation of pain.

Our study results clearly indicate that insulin has its role in the modulation of pain involving opioid pathway. The results of the protein - protein docking method further evidence the possible interaction between insulin and opioid receptors, more specifically, the kappa receptor through hydrogen bonding; however, the active site of either receptor or the residues of insulin involved in binding could not be clearly established. To substantiate this, the results of the study showed significant increase in insulin level in pentazocine induced antinociception as compared to control and therefore it is suggested that insulin involves in the pain threshold utilizing opioid mechanism through kappa receptors.

7.6 Mechanisms of diurnal rhythm on association between endogenous insulin and pain threshold

The diurnal variations in the pain intensity as well as in antinocieptive response induced by opioid drugs have been documented. Endorphins and enkephalins are endogenous peptides found in the region of the brain known to be involved in the perception of pain and their brain concentrations have been shown to fluctuate over a 24 hour period. Time-dependent changes have also been detected in the concentrations of beta endorphin in the pituitary gland, the pons, the medulla and the cerebellum of the rat brain. Peak levels of this peptide always occurred during

the activity period of the animals.[163] The diurnal variation of opioid receptors binding and the observed differences in binding influenced by circadian rhythm is reportedand suggest that the difference of binding throughout the day was due to the changes in the number of binding sites rather than in the affinity of the drug for the receptor site in a study conducted in rats.[210]

The mechanisms of the time-dependent variation in the action of morphine and its related drugs on the central nervous system involving the adrenergic, serotonergic or cholinergic system have been reported.[149] One study conducted in rats reports morphine in small dose significantly raised total brain acetyl choline content in the active period in the rats whereas a large dose was needed to get the same effect in the resting period.[169] In the present study, the role of opioid mechanism on the involvement of endogenous insulin in the modulation of pain was again tested through diurnal effect in mice using high dose naloxone, an opioid antagonist. High dose naloxone in dark as well as light cyle reversed the elevated serum insulin level significantly to the control value (nociceptive stimuli value) in association with significant reduction in antinociceptive action of pentazocine independent of the glycemic status in acetic acid, formalin and tail flick pain models. The reversal effects were more in the dark cycle whereas least in the light cycle. This observation tends to suggest the diurnal rhythm of opioid mechanism on the involvement of endogenous insulin in the modulation of pain. Therefore, it can be clearly stated that though there exists relation between endogenous insulin and pain threshold there are diurnal variations in endogenous insulin level and pain intensity.

8. SUMMARY AND CONCLUSION

- The present study investigated the relation between endogenous insulin level and pain threshold and its interaction with opioid system and diurnal rhythm in animal model using different nociceptive tests and pentazocine as a model drug.

- We observed significant relation between endogenous insulin level and pain threshold independent of the glycemic status in acetic acid, formalin and tail flick models except in the hot plate model where such a relation seems dependent on the glycemic status.

- Insulin appears interacting with opioid receptor (kappa receptor) based on the preliminary study by protein-protein docking method.

- Endogenous insulin positively correlate with pain threshold which was found to be influenced by diurnal rhythm.

- Endogenous insulin level showed peak in the dark cycle and trough in the light cycle in all the pain models investigated.

- The study reports for the first time on the involvement of endogenous insulin in pain threshold against different nociceptive tests in animal models.

The above findings clearly delineate that there seems to be no direct relation between blood glucose levels and antinociception, however, a possible involvement of endogenous insulin in pain threshold through opioid pathway by binding with kappa receptor which is independent of the glycemic status. The association between endogenous insulin and pain threshold seems dependent on the type of nociceptive stimuli and the endogenous insulin level and pain intensities seems influenced by diurnal rhythm.

9. RECOMMENDATIONS

The present study clearly reveals the significant role of endogenous insulin in the modulation of pain utilizing opioid pathway. Our preliminary study on the interaction between endogenous insulin and opioid receptor show possible binding of insulin on kappa receptor. Future study is recommended to explore the mechanisms of insulin mediated antinociception through opioid pathway at molecular level.

10. REFERENCES

1. Rocha AP, Kraychete DC, Lemonica L, Carvalho LR et al. Pain: Current aspects on peripheral and central sensitization. Rev Bras Anestesiol 2007;57:94-05.

2. Bruguerolle B, Labrecque G. Rhythmic pattern in pain and their chronotherapy. Adv Drug Deliv Rev 2007;59:883-95.

3. Wesche DL, Frederickson RCA. Diurnal differences in opioid peptide levels correlated with nociceptive sensitivity. Life Sci 1979;24:1861-67.

4. Davis WM, Miya TS, Edwards LD. The influence of glucose and pretreatment upon morphine analgesia in rat. J Am Pharm Assoc 1956;45:60-2.

5. Takeshita N, Yamaguchi I. Insulin Attenuates formalin induced nociceptive response in mice through a mechanism that is deranged by diabetes mellitus. J Pharmacol Exp Ther 1997;7:281-85.

6. Schulingkamp RJ, Pagano TC, Hung D, Raffa RB. Insulin receptors and insulin action in the brain: review and clinical implications. Neurosci Biobehav Rev 2000;24:855-72.

7. Wisniewski K, Zarebski M. Effect of Insulin on the transport and the analgesic action of sodium salicylates. Metabolism 1968;17:212-7.

8. Singh IS, Chatterjee TK, Ghosh JJ. Modification of morphine antinociceptive response by blood glucose status: Possible involvement of cellular energetics. Eur J Pharmacol 1983;90:437-9.

9. Simon GS, Dewey WL. Narcotics and diabetes. I. The effects of streptozotocin-induced diabetes on the antinociceptive potency of morphine. J Pharmacol Exp Ther 1981;218:318-23.

10. Simon GS, Borzelleca J, Dewey WL. Narcotics and diabetes. II. Streptozotocin-induced diabetes selectively alters the potency of certain narcotic analgesics. Mechanism of diabetes: Morphine interaction. J Pharmacol Exp Ther 1981;218:324-9.

11. Priano L, Miscio G, Grugni G, Milano E et al. On the origin of sensory impairment and altered pain perception in Prader-Willi syndrome: a neurophysiological study. Eur J Pain 2009;13:829-35.

12. Delaney CA, Mouser JV, Westerman RA. Insulin sensitivity and sensory nerve function. Clin Exp Neurol 1994;31:19-37.

13. Rajendran NN, Thirugnanasambandam P, Parvathavarthini S, Viswanathan S et al. Modulation by insulin rather than blood glucose of the pain threshold in acute Physiological and flavone induced antinociception in mice. Indian J Exp Biol 2001;39:1009-16.

14. Reid RL, Yen SSC. Beta endorphin stimulates the secretion of insulin and glucagon in humans. J Clin Endocrinol and Metab 1981;52:592-4.

15. Pierluissi R, Pierluissi J, Ashcroft SJH. Effects of an enkephalin analogue (DAMME) on insulin release from cultured rat islets of Langerhans. Diabetologia 1981;20:642-6.

16. Ipp E, Dobbs RH, Unger RH. Morphine and B-endorphin influence the secretion of endocrine pancreas. Nature 1978;276:190-1.

17. Sahley TL, Nodar RH. Improvement in auditory function following pentazocine suggests a role for dynorphins in auditory sensitivity. Ear Hear 1994;6:422-31.

18. Verspohl EJ, Berger U, Ammon HP. The significance of mu and delta receptors in rat pancreatic islets for the opioid-mediated insulin release. Biochim Biophys Acta 1986;888:217-24.

19. Curry DL, Bennett LL, Li CH. Stimulation of Insulin secretion by beta endorphins. Life Sci 1987;40:2053-8.

20. Loh HH, Tseng LF, Wei E, Li CH. Beta endorphin is a potent analgesic agent. Proc Natl Acad Sci U S A. 1976;73:2895-898.

21. Levine JD, Gordon NC, Taiwo YO, Coderre TJ. Potentiation of pentazocine analgesia by low-dose naloxone. J Clin Invest 1988;82:1574-7.

22. Labrecque G, Vaniert MC. Biological rhythms in pain and in the effects of opioid analgesics. Pharmac Ther 1995;68:129-47.

23. Morris RW, Lutsch EF. Susceptibility to morphine-induced analgesia in mice. Nature 1967;216:494-5.

24. Mejean L, Bicakova-Rocher A, Kolopp M, Villaume C et al. Circadian and ultradian rhythms in blood glucose and plasma insulin of healthy adults. Chronobiol Int 1988;5:227-36.

25. Pessacq MT, Mercer RG, Rebolledo OR, Gagliardino JJ. Effect of fasting on the circadian rhythm of serum insulin levels. Chronobiologia 1976;3:20-6.

26. Boden G, Ruiz J, Urbain JL, Chen X. Evidence for a circadian rhythm of insulin secretion. Am J Physiol 1996;271:E246-E52.

27. Hellman D. Circadian and ultradian rhythms in blood glucose and plasma insulin of healthy adults. Chronobiol Int 1988;5:227-36.

28. Wood S. Special focus. Pain. Nurs Times 2002;98:41-4

29. Grichnik KP, Ferrante FM. The difference between acute and chronic pain. Mt Sinai J Med 1991;58:217-20.

30. Dunwoody CJ, Krenzischek DA, Pasero C, Rathmell JP et al. Assessment, physiological monitoring, and consequences of inadequately treated acute pain. J Perianesth Nurs 2008;23:S15-7.

31. McCaffery M, Pasero C. Assessment: Underlying complexities, misconceptions, and practical tools. In: McCaffery M, Pasero C, editors. Pain Clinical Manual. St. Louis: Mosby Inc; 1999. p. 35-102.

32. Serpell GM, Makin A, Harvey A. Acute pain physiology and pharmacological targets, the present and future. Acute Pain 1998;1:31-47.

33. Dray A. Tasting the inflammatory soup: The role of peripheral neurones. Pain Rev 1994;1:153-71.

34. Mao J. NMDA and opioid receptors: their interactions in antinociception, tolerance and neuroplasticity. Brain Res Rev 1999;30:289-4.

35. Snyder SH, Pasternak GW. Historical review: Opioid receptors. Trends Pharmacol Sci 2003;24:198-5.

36. Chretien M, Seidah NG, Scherrer H. [Endorphins: structure, roles and biogenesis]. Can J Physiol Pharmacol 1981;59:413-31.

37. Partin C. Runner's `High'. JAMA 1983;249:21.

38. Chapman CL, De Castro JM. Running addiction: measurement and associated psychological characteristics. J Sports Med Phys Fitness 1990;30:283-90.

39. Sprenger T, Berthele A, Platzer S, Boecker H et al. What to learn from in vivo opioidergic brain imaging?. Eur J Pain 2005;9:117-21.

40. Dalayeun JF, Norès JM, Bergal S. Physiology of beta-endorphins. A close-up view and a review of the literature. Biomed Pharmacother 1993;47:311-20.

41. Comb M, Seeburg PH, Adelman J, Eiden L et al. Primary structure of the human Met- and Leu-enkephalin precursor and its mRNA. Nature 1982;295:663-6.

42. Day R, Lazure C, Basak A, Boudreault A et al. Prodynorphin processing by proprotein convertase 2. Cleavage at single basic residues and enhanced processing in the presence of carboxypeptidase activity. J Biol Chem 1998;273:829-36.

43. Holden JE, Jeong Y, Forrest JM. The endogenous opioid system and clinical pain management. AACN Clin Issues. 2005;16:291-301.

44. Basbaum AI, Fields HL. Endogenous pain control mechanisms: review and hypothesis. Ann Neurol 1978;5:451-62.

45. Przewlocki R, Przewlocka B. Opioids in chronic pain. Eur J Pharmacol 2001:429:79-91.

46. Suh HH, Fujimoto JM, Tseng LF. Different radiant heat intensities differentiate intracerebroventricular morphine- from beta-endorphin-induced inhibition of the tail-flick response in the mouse. Eur J Pharmacol 1992;213:337-41.

47. Hughes J, Smith TW, Kosterlitz HW, Fothergill LA et al. Identification of two related pentapeptides from the brain with potent opiate agonist activity. Nature 1975;258:577-80.

48. Guillemin, Roger Vargo, Therese Rossier, Jean Minick et al. Beta-endorphin and adrenocorticotropin are secreted concomitantly by the pituitary gland. Science 1977;197:1367-9.

49. Shiomi H, Watson SJ, Kelsey JE, Akil H. Pretranslational and posttranslational mechanisms for regulating beta-endorphin-adrenocorticotropin of the anterior pituitary lobe. Endocrinology 1986;119:1793-9.

50. Borders JL. New pituitary peptide relationships. South Med J 1980;73:473-6.

51. Grube D, Voight KH, Weber E. Pancreatic glucagon cells contain endorphin-like immunoreactivity. Histochemistry 1978;59:75-9.

52. Bruni JF, Watkins WB, Yen SSC: Beta-endorphin in the human pancreas. J Clin Endocrinol Metab 1979;49:649-51.

53. Polak JM, Bloom SR, Sullivan SN, Facer P. Enkephalin-like immunoreactivity in the human gastrointestinal tract. Lancet 1977;1:972-74.

54. Sachse G, Laube H, Svedberg J, Breidenbach Th et al. Opiates influence endocrine pancreas secretion. Abstract Diabetologia 1981;21:322A.

55. Corbett AD, Paterson SJ, McKnight AT, Magnan J et al. Dynorphin-(1-8) and dynorphin-(1-9) are ligands for the k subtype of opiate receptor. Nature 1982;299:79-81.

56. Hermansen K. Enkephalins and the secretion of pancreatic somatostatin and insulin in the dog: studies in vitro. Endocrinology 1983;113:1149-54.

57. Rudman D, Berry CJ, Riedeburg CH, Hollins BM et al. Effects of opioid peptides and opiate alkaloids on insulin secretion in the rabbit. Endocrinology 1983;112:1702-10.

58. Green IC, Perrin D, Pedley KC, Leslie RDG et al. Effects of enkephalins and morphine on insulin secretion from isolated rat islets. Diabetologia 1980;19:158-61.

59. Kanter RA, Ensinck JW, Fujimoto WY. Disparate effects of enkephalin and morphine upon insulin and glucagon secretion by islet cell cultures. Diabetes 1980;29:84-6.

60. Adeghate E, Ponery AS. The role of leucine-enkephalin on insulin and glucagon secretion from pancreatic tissue fragments of normal and diabetic rats. Arch Physiol Biochem 2001;109:223-9.

61. Green IC, Ray K, Perrin D. Opioid peptide effects on insulin release and c-AMP in islets of Langerhans. Horm Metab Res1983;15:124-8.

62. García-Barrado MJ, Iglesias-Osma MC, Rodríguez R, Julio Moratinos MM. Role of μ-opioid receptors in insulin release in the presence of inhibitory and excitatory secretagogues. E J Pharmacol 2002;448:95–104.

63. Pfeiffer A, Herz A. Endocrine Actions of Opioids. Horm Metab Res 1984;16:386-97.

64. Ipp E, Schusdiarra V, Harris V, Unger RH. Morphine induced hyperglycaemia: role of insulin and glucagon. Endocrinology 1980;107:461-3.

65. Schleicher RL, Chawla RK, Coan PA, Saltzman M et al. Beta-endorphin-induced hyperglycemia in rabbits: effects of a glucose or arginine challenge. Am J Physiol 1987;252:E255-9.

66. Ipp E, Dhorajiwala J, Pugh W, Moossa AR et al. Effects of an enkephalin analog on pancreatic endocrine function and glucose homeostasis in normal and diabetic dogs. Endocrinology 1982;111:2110-6.

67. Watkins WB, Bruni JR and Yen SSC. B-endorphin and somatostatin in the pancreatic D-cell. Co-localisation by immunocytochemistry. J Histochem Cytochem 1980;28:1170-4.

68. Matsumura M, Fukushima T, Saito H, Saito S. In vivo and in vitro effects of beta-endorphin on glucose metabolism in the rat. Horm Metab Res 1984,16:27-31.

69. Ahren B. Effects of beta-endorphin, met-enkephalin, and dynorphin A on basal and stimulated insulin secretion in the mouse. Int J Pancreatol 1989;5:165-78.

70. Feldman M, Kiser RS, Unger RH, Li CH. Beta-Endorphin and the Endocrine Pancreas - Studies in Healthy and Diabetic Human Beings. N Engl J Med 1983;308:349–53.

71. Giugliano D. Morphine, opioid peptides and pancreatic islet function. Diabetes Care 1984;7:92-8.

72. Bell GI, Picket RL, Rutter WJ et al. Sequence of the human insulin gene. Nature 1980;284:26-32.

73. Subramaniam S, Madhavankutty K, Singh HD. Text book of Human physiology. 6th ed. New Delhi: S.Chand & Company Ltd; 2001.

74. Steiner DF, Rouille Y, Gong Q et al. The role of prohormone convertases in insulin biosynthesis: Evidence for inherited defects in their action in man and experimental animals. Diabetes Metab 1996;22:94-104.

75. Sharma HL, Sharma KK. Priniciples of Pharmacology. 1st ed. New Delhi: Paras Medical Publisher; 2007.

76. Tripathi KD. Essential of medical pharmacology. 5th ed. New Delhi: Jaypee publishers; 2003.

77. Matchinsky FM, Banting Lecture. A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. Diabetes 1996;45:223-41.

78. Laurence LB, Lazo JS, Parker KL. Goodman & Gilman's, The Pharmacological basis of Therapeutics. 11th ed. New Delhi: MC Graw Hill; 2006.

79. Virkamaki A, Ueki K, Kahn CR. Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. J Clin Invest 1999:103:931-43.

80. Conlon JM, Reinecke M, Thomdyke MC, Falmer S. Insulin and other islet hormones (somatostatin glucagon and PP) in the neuroendocrine system of some lower vertebrates and that of invertebrates---a mini review. Horm Metab Res 1988;20:406-10.

81. Ebberink RHM, Joosse J. Molecular properties of various snail peptides from brain and gut. Peptides 1985:6:451-57.

82. Girbau M, Gomez JA, Lesniak MA, de Pablo F. Insulin and insulin-like growth factor I both stimulate metabolism, growth and differentiation in the postneurula chick embryo. Endocrinology 1987;121:1477-82.

83. Ada T, Takiya S, Suzuki Y, Iwami M et al. cDNA structure and expression of bombyxin, an insulin-like brain secretory peptide of the silkmoth Bombyx mori. J Boil Chem 1989;264:7681-7685.

84. Schechter R, Holtzclaw L, Sadiq F, Kahn A et al. Insulin synthesis by isolated rabbit neurons. Endocrinology 1988;123:505-13.

85. Smit AB, Ebberink RHM, Joosse J. Molluscan insulin-related peptides from the brain and the digestive tract of the snail Lymnaea stagnalis. J Mol Endocrinol 1993;11:103-13.

86. Havrankova J, Roth J, Brownstein M. Insulin receptors are widely distributed in the central nervous system of the rat. Nature 1978;272:827-9.

87. Pacold ST, Blackard WG. Central nervous system insulin receptors in normal and diabetic rats. Endocrinology 1979;105:1452-7.

88. Raizada MK, Yang JW, Fellows RE. Binding of (125-1)-insulin to specific receptors and stimulation of nucleotide incorporation in cells cultured from rat brain. Brain Res 1980;200:389-400.

89. Heidenreich KA, Zahniser NR, Berhanu P, Brandenburg D et al. Structural differences between insulin receptors in the brain and peripheral target tissues. J Biol Chem 1983;258:8527-30.

90. Lowe WL Jr, Boyd FT, Clarke DW, Raizada MK et al. Development of brain insulin receptors: structural and functional studies of insulin receptors from whole brain and primary cell cultures. Endocrinology 1986;119:25-35.

91. Gerozissis K. Brain insulin, energy and glucose homeostasis; genes, environment and metabolic pathologies. Eur J Pharmacol 2008;585:38-49.

92. Baskin DJ, Porte Jr D, Guest K, Dorsa DM. Regional concentrations of insulin in the rat brain. Endocrinology 1983;112:898–903.

93. Bruning JC, Gautam D, Burks DJ, Gillette J et al. Role of brain insulin receptor in control of body weight and reproduction. Science 2000;289:2122-25.

94. Gerozissis K. Brain insulin: regulation, mechanisms of action and functions. Cell Mol Neurobiol 2003;23:1-25.

95. Havrankova JM, Roth J, Brownstein M. Concentrations of insulin and of insulin receptors in the brain are independent of peripheral insulin levels. Studies of obese and streptozotocin-treated rodents. J Clin Invest 1979;64:636-42.

96. Schechter R, Beju D, Miller KE. The effect of insulin deficiency on tau and neurofilament in the insulin knockout mouse. Biochem Biophys Res Commun 2005;334:979-86.

97. Schwartz MW, Figlewicz DP, Baskin DG, Woods SC et al. Insulin in the brain: a hormonal regulator of energy balance. Endocr Rev 1992;13:387-414.

98. Zhao WQ, Alkon DL. Role of insulin and insulin receptors in learning and memory. Mol Cell Endocrinol 2001:177:125-34.

99. Wozniak M, Rydzewski B, Baker SP, Raizada MK. The cellular and physiological actions of insulin in the central nervous system. Neurochem Int 1993;22:1-10.

100. Banks WA. The source of cerebral insulin. Eur J Pharmacol 2004;490:5-12.

101. Baura GD, Foster DM, Porte Jr D, Kahn SE et al. Saturable transport of insulin from plasma into the CNS of dogs in vivo. A mechanism for regulated insulin delivery to the brain. J Clin Invest 1993;92:1824-30.

102. Woods SC, Seeley RJ, Baskin DG, Schwartz MW. Insulin and the blood-brain barrier. Curr Pharm Des 2003;9:795-800.

103. Clarke DW, Mudd L, Boyd FT, Fields M et al. Insulin is released from rat brain neuronal cells in culture. J Neurochem 1986;47:831-6.

104. Devaskar SU, Giddins SJ, Rajakumar PA, Canaghi LR et al. Insulin gene expression and insulin synthesis in mammalian neuronal cells. J Biol Chem 1994;269:8445-54.

105. Dorn A, Bernstein HG, Hahn HJ, Ziegler M et al. Insulin immunohistochemistry of rodent CNS: apparent species differences but good correlation with radioimmunological data. Histochemistry 1981;71:609-16.

106. Grunblatt E, Salkovic-Petrisic M, Osmanovic J, Riederer P et al. Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. Neurochemistry 2007;101:757-70

107. Hrytsenko O, Wright Jr JR, Morrison CM, Pohajdak B. Insulin expression in the brain and pituitary cells of tilapia (Oreochromis niloticus). Brain Res 2007;1135:31–40.

108. Schechter R, Sadiq HF, Devaskar SU. Insulin and insulin mRNA are detected in neuronal cell cultures maintained in an insulin-free/serum-free medium. J Histochem Cytochem 1990;38:829-36.

109. Schechter R, Whitmire J, Wheet GS, Beju D et al. Immunohistochemical and in situ hybridization study of an insulin-like substance in fetal neuron cell cultures. Brain Res 1994;636:9-27.

110. Schechter R, Beju D, Gaffney T, Schaefer F. Preproinsulin I and II mRNAs and insulin electron microscopic immunoreaction are present within the rat fetal nervous system. Brain Res 1996;736:16-27.

111. Steen E, Terry BM, Rivera EJ, Cannon JL et al. 2005. Impaired insulin and insulin-like growth factor expression and signalling mechanisms in Alzheimer's disease is this type 3 diabetes? J Alzheimers 2005;7:63-80.

112. Coleman DL, Hummel KP. Studies with the mutation, diabetes, in the mouse. Diabetologia 1967;3:238-48.

113. Kodama H, Fujita M, Yamazaki M, Yamaguchi I. The possible role of age-related increase in the plasma glucagon/insulin ratio in the enhanced hepatic gluconeogenesis and hyperglycemia in genetically diabetic (C57BL/ KsJ-db/db) mice. Jpn J Pharmacol 1994;66:281-7.

114. Hajializadeh Z, Esmaeili-Mahani S, Sheibani V, Kaeidi A et al. Changes in the gene expression of specific G-protein subunits correlate with morphine insensitivity in streptozotocin-induced diabetic rats. Neuropeptides 2010;44:299-304.

115. Kamei J, Sodeyama M, Kimura M, Tanaka S. Modulation by serum glucose levels to morphine-induced antinociceptive effect in C57BLrKsJ-dbrdb mice. Life Sci 1998;62:PL1–6.

116. Fujiwara T. Yoshioka S, Yoshioka T, Ushiyama I et al. Characterization of CS-045, a new oral antidiabetic agent CS-045. Studies in KK and db/db mice and Zuker Fatty rats. Diabetes 1988:37:1549-58.

117. Fujiwara T, Wada M, Fukuda K, Fukami M et al. Characterization of CS-045, a new oral antidiabetic agent: II. Effects on glycemic control and pancreatic islet structure at a late stage of the diabetic syndrome in C57BLrKsJ-dbrdb mice. Metabolism 1991;40:1213-8.

118. Kamei J, Ohsawa M, Sodeyama M, Kimura M et al. Intraventricular insulin reduces the antinociceptive effect of [D-Ala2, NMePhe4, Gly-ol5] enkephalin in mice. E J of Pharmacol 1998;345:171-4.

119. Anuradha K, Hota D, Pandhi P. Possible mechanisms of insulin antinociception. Methods Find Exp Clin Pharmacol 2004;26:5-8.

120. Venkataramanan PE, Parvathavarthini S, Viswanathan S, Ramaswamy S. Role of ATP sensitive potassium channel on 7-hydroxy flavone induced antinociception and possible association with changes in glycaemic status. Indian J Exp Biol 2000;38:1172-4.

121. Singleton JR. Smith AG, Bromberg MB. Painful sensory polyneuropathy associated with impaired glucose tolerance. Muscle Nerve 2001;24:1225-8.

122. Chan AW, MacFarlane IA, Bowsher D. Short term fluctuations in blood glucose concentrations do not alter pain perception in diabetic patients with and without painful peripheral neuropathy. Diabetes Res 1990;14:15-9.

123. Thye-Ronn P, Sindrup SH, Arendt-Nielsen L, Brennum J et al. Effect of short-term hyperglycemia per se on nociceptive and non-nociceptive thresholds. Pain 1994;56:43-9.

124. Courteix C, Bardin M, Massol J, Fialip J et al. Daily insulin treatment relieves long-term hyperalgesia in streptozocin diabetic rats. Neuroreport 1996;7:1922-4.

125. Dobretsov M, Hastings SL, Romanovsky D, Stimers JR et al. Mechanical hyperalgesia in rat models of systemic and local hyperglycemia. Brain Res 2003;960:174-83.

126. Maneuf YP, Blake R, Andrews NA, McKnight AT. Reduction by gabapentin of K+-evoked release of [3H]-glutamate from the caudal trigeminal nucleus of the streptozotocin treated rat. Br J Pharmacol 2004;141:574-9.

127. Romanovsky D, Hastings SL, Stimers JR, Dobretsov M. Relevance of hyperglycemia to early mechanical hyperalgesia in streptozotocin-induced diabetes. J Peripher Nerv Syst 2004;9:62-9.

128. Brussee V, Cunningham FA, Zochodne DW. Direct insulin signaling of neurons reverses diabetic neuropathy. Diabetes 2004;53:1824-30.

129. Huang TJ, Price SA, Chilton L, Calcutt NA et al. Insulin prevents depolarization of the mitochondrial inner membrane in sensory neurons of type 1 diabetic rats in the presence of sustained hyperglycemia. Diabetes 2003;52:2129-36.

130. Schmidt RE, Dorsey DA, Beaudet LN, Parvin CA et al. Experimental rat models of types 1 and 2 diabetes differ in sympathetic neuroaxonal dystrophy. J Neuropathol Exp Neurol 2004;63:450-60.

131. Romanovsky D, Nancy FC, Gerald AD, Dobretsov M. Mechanical hyperalgesia correlates with insulin deficiency in normoglycemic streptozotocin-treated rats. Neurobiol Dis 2006;24:384-94.

132. Dobretsov M, Hastings SL, Romanovsky D, Stimers JR et al. Mechanical hyperalgesia in rat models of systemic and local hyperglycemia. Brain Res 2003;960:174-83.

133. Saini AK, Arun KHS, Kaul CL, Sharma SS. Acute hyperglycemia attenuates nerve conduction velocity and nerve blood flow in male Sprague–Dawley rats: reversal by adenosine. Pharmacol Res 2004;50:593-9.

134. Biessels NA, Cristino GJ, Rutten FPT, Hamers DW et al. Neurophysiological changes in the central and peripheral nervous system of streptozotocin diabetic rats. Course of development and effects of insulin treatment. Brain 1999;122:757-68.

135. Calcutt NA, Jorge MC, Yaksh TL, Chaplan SR. Tactile allodynia and formalin hyperalgesia in streptozotocin-diabetic rats: effects of insulin, aldose reductase inhibition and lidocaine. Pain 1996;68:293-9.

136. S.Srinivasan S, Stevens M, Wiley JW. Diabetic peripheral neuropathy. Evidence for apoptosis and associated mitochondrial dysfunction. Diabetes 2000;49:1932-8.

137. Kumar S, Arun KH, Kaul CL, Sharma SS. Effects of adenosine and adenosine A2A receptor agonist on motor nerve conduction velocity and nerve blood flow in experimental diabetic neuropathy. Neurol 2005;27:60-6.

138. Ishii DN. Implication of insulin-like growth factors in the pathogenesis of diabetic neuropathy. Brain Res Brain Res Rev 1995;20:47-67.

139. Recio-Pinto E, Rechler MM, Ishii DN. Effects of insulin, insulin-like growth factor-II, and nerve growth factor on neurite formation and survival in cultured sympathetic and sensory neurons. J Neurosci 1986;6:1211-9.

140. Berti-Mattera L, Peterson R, Bel M, Eichberg J. Effect of hyperglycemia and its prevention by insulin treatment on the incorporation of 32P into polyphosphoinositides and other phospholipids in peripheral nerve of the streptozotocin diabetic rat. J Neurochem 1985;45:1692-8.

141. Hoybergs YM, Meert TF. The effect of low-dose insulin on mechanical sensitivity and allodynia in type I diabetes neuropathy. Neurosci Lett 2007;417:149-4.

142. Sugimoto K, Rashid IB, Shoji M, Suda T et al. Early changes in insulin receptor signaling and pain sensation in streptozotocin-induced diabetic neuropathy in rats. J Pain 2008;9:237-45.

143. Visnagri A, Kandhare AD, Chakravarty S, Ghosh P, Bodhankar SL. Hesperidin, a flavanoglycone attenuates experimental diabetic neuropathy via modulation of cellular and biochemical marker to improve nerve functions. Pharm Biol 2014 Feb 21. (Epub ahead of print)

144. Pabbidi RM, Yu SQ, Peng S, Khardori R et al. Influence of TRPV1 on diabetes-induced alterations in thermal pain sensitivity. Mol Pain 2008;4:9.

145. Ohsawa M, Miyata S, Carlsson A, Kamei J. Preventive effect of acetyl-L-carnitine on the thermal hypoalgesia in streptozotocin-induced diabetic mice. Eur J Pharmacol 2008;588:213-6.

146. Kolta MG, Ngong JM, Rutledge LP, Pierzchala K et al. Endogenous opioid peptide mediation of hypoalgesic response in long term diabetic rats. Neuropeptides.1996;30:335-44.

147. Williams JT, Christie MJ, Manzoni O. Cellular and synaptic adaptations mediating opioid dependence. Physiol Rev 2001;81:299-343.

148. Ahren B, Ericson LE, Lundquist I, Lorén I et al. Adrenergic innervation of pancreatic islets and modulation of insulin secretion by the sympatho-adrenal system. Cell Tissue Res 1981;216:15-30.

149. Danzebrink, RM, Gebhart GF. Antinociceptive effects of intrathecal adrenoreceptor agonists in a rat model of visceral nociception. J Pharmacol Exp Ther 1990;253:698-5.

150. Meert TF, Dekock M. Potentiation of the analgesic propertiesof fentanyl-like opioids with a2-adrenoreceptor agonists in rats. Anaesthesiology 1994;81:677-88.

151. Mendez R, Eisenach JC, Kashtan K. Epidural clonidine analgesia after caesarian section. Anaesthesiology 1990;73:848-52.

152. Millan MJ, Bervoets K, Rivet JM, Widdowson P et al. Multiple Alpha 2 adrenergic receptors in the control of nociception, motor behaviour and hippocampal synthesis of noradrenaline. J Pharmacol Exp Ther 1994;270:958-72.

153. Monasky MS, Zinsmeister AR, Stevens CW, Yaksh TL. Interaction of intrathecal morphine and ST91 on antinociception in the rat: Dose-response analysis, antagonism and clearance. J Pharmacol Exp Ther 1990;254:383-92.

154. Milad S, Charles WTP. Insulin-Dependent Attenuation in alpha 2-Adrenoreceptor–Mediated Nociception in Experimental Diabetes. Pharmacol Biochem Behav 1997;56:15-20.

155. Pan M, Yang G, Cui X, Yang SN. Subthreshold α2-adrenergic activation counteracts glucagon-like peptide-1 potentiation of glucose-stimulated insulin secretion. Exp Diabetes Res 2011;2011:604989.

156. Junker U, Wirz S. Chronobiology: influence of circadian rhythms on the therapy of severe pain. J Oncol Pharm Pract 2010;16:81-7.

157. Aronson BD, Bell-Pedersen D, Block GD, Bos NP et al. Circadian rhythms. Brain Res Brain Res Rev 1993;18:315-33.

158. Duffield GE. DNA microarray analyses of circadian timing: the genomic basis of biological time. J Neuroendocrinol 2003;15:991-02.

159. Schibler U, Sassone-Corsi P. A web of circadian pacemakers. Cell 2002;111:919-2.

160. Klerman EB, Elizabeth. Clinical aspects of human circadian rhythms. J Biol Rhythms 2005;20:375-86.

161. Belanger PM, Bruguerolle B, Labrecque . Rhythms in Pharmacokinetics: absorption, distribution, metabolism and excretion. In: Redfern PH, Lemmer B, editors, Physiolgy and pharmacology of Biological Rhythms. Hand book of Experimental Pharmacology. Berlin: Springer-Verlag Inc; 1997.

162. Hidebrandt G, Moser M, Lehofer M. Chronobiologie and chronomedizin. Stuttgart: Hippokrates Verlag; 1998.

163. Frederickson RC, Burgis V, Edwards JD. Hyperalgesia induced by naloxone follows diurnal rhythm in responsivity to painful stimuli. Science 1977;198:756-8.

164. Kavaliers M, Hirst M. Daily rhythms of analgesia in mice: effects of age and photoperiod. Brain Res 1983;279:387-3.

165. Konecka AM, Sroczynska I. Circadian rhythm of pain in male mice. Gen Pharmacol 1998;31:809-10.

166. Kohichi S, Masashi A, Masami O, Koh-ichi S et al. Daily variation in an intensity of kaolin - induced writhing reaction in mice. Life Sci 1997;61:1253-9.

167. Yoshida M, Ohdo S, Takane H, Tomiyoshi Y et al. Chronopharmacology of analgesic effect and its tolerance induced by morphine in mice. Pharmacol Exp Ther 2003;305:1200-5.

168. Ohdo N, Ogawa N, Song JG, Higuchi S. Chronopharmacological study of KE-SI-TO components in mice. Life Sci 1998;62:2057-4.

169. Labrecque G, Domino EF. Temporal variations in the effect of morphine and nalorphine on total brain acetylcholine content in the rat. Arch Int Pharmacodyn Ther 1982;259:244-9.

170. Martinez-Gomez M, Cruz Y, Salas M, Hudson R et al. Assessing pain threshold in the rat: changes with estrus and time of day. Physiol Behav 1994;55:651-7.

171. Cannon CP, McCabe CH, Stone PH, Schactman M, et al. Circadian variation in the onset of unstable angina and non-Q-wave acute myocardial infarction (the TIMI III Registry and TIMI IIIB). Am J Cardiol 1997;79:253-8.

172. Pollmann L. Circadian changes in the duration of local anaesthesia. Int J Oral Surg 1982;11:36-9.

173. Orr WC. Night-time gastro-oesophageal reflux disease: prevalence, hazards, and management. Eur J Gastroenterol Hepatol 2005;17:113-20.

174. Kowanko ICR, Pownall R, Knapp MS, Swannel AJ et al. Circadian variations in the signs and symptoms of rheumatoid arthritis and in the therapeutic effectiveness of flurbiprofen at different times of the day. Br J Clin Pharmacol 1981;11:477-84.

175. Cutolo M, Masi AT. Circadian rhythms and arthritis. Rheum Dis Clin North Am 2005;31:115-29.

176. Solomon GD. Circadian rhythms and migraine. Cleve Clin J Med 1992;59:326-9.

177. Koch HJ, Raschka C. Diurnal variation of pain perception in young volunteers using the tourniquet pain model. Chronobiol Int 2004;21:171-3.

178. Rigas B, Torosis J, McDougall CJ, Vener KJ et al. The circadian rhythm of biliary colic. J Clin Gastroenterol 1990;12:409-4.

179. Bruera E, Macmillan K, Kuehn N, Miller MJ. Circadian distribution of extra doses of narcotic analgesics in patients with cancer pain: a preliminary report. Pain 1992;49:311-4

180. Gobel H, Cordes P. Circadian variation of pain sensitivity in pericranial musculature. Headache 1990;30:418-22.

181. Manfredini R, Gallerani M, Cecilia O, Boari B et al. Circadian pattern in occurrence of renal colic in an emergency department: analysis of patients' notes. BMJ 2002;324:767.

182. Boari B, Manfredini R. [Circadian rhythm and renal colic]. Recenti Prog Med 2003;94:191-3.

183. Pollmann L. Duality of pain demonstrated by the circadian variation in tooth sensitivity. In: Haus E, Kabat H, editors. Chronobiology. Basel: Karger; 1984. p. 225-228.

184. Aya AG, Vialles N, Mangin R, Robert C, et al. Chronobiology of labour pain perception: an observational study. Br J Anaesth 2004;93:451-3.

185. Muller JE, Ludmer PL, Willich SN, Tofler GH, et al. Circadian variation in the frequency of sudden cardiac death. Circulation 1987;75:131-8.

186. Kerdelhue B, Palkovits M, Karteszi M, Reinberg A. Circadian variations in substance P, luliberin (LH-RH) and thyroliberin (TRH) contents in hypothalamic and extra hypothalamic brain nuclei of adult male rats. Brain Res 1981;206:405-13.

187. Tang F, Tang J, Chou J, Costa E. Age-related and diurnal changes in Met5-Enk-Arg6-Phe7 and Met5-enkephalin contents of pituitary and rat brain structures. Life Sci 1984;35:1005-14.

188. Barni S, Lissoni P, Rovelli F, Crispino S et al. Alteration of opioid peptide circadian rhythm in cancer patients. Tumori 1988;74:357-60.

189. Pikula DL, Harris EF, Desiderio DM, Fridland GH et al. Methionine enkephalin-like, substance P-like, and beta-endorphin-like immunoreactivity in human parotid saliva. Arch Oral Biol 1992;37:705-9.

190. Leone M, Lucini V, D'Amico D, Moschiano F et al. Twenty-four-hour melatonin and cortisol plasma levels in relation to timing of cluster headache. Cephalalgia 1995;15:224-9.

191. Hindmarsh KW, Tan L, Sankaran K, Laxdal VA. Diurnal rhythms of cortisol, ACTH and beta endorphin levels in neonates and adults. West J Med 1989;151:153-6.

192. Shavali S, Ho B, Govitrapong P, Sawlom S et al. Melatonin exerts its analgesic actions not by binding to opioid receptor subtypes but by increasing the release of beta-endorphin an endogenous opioid. Brain Res Bull 2005;64:471-9.

193. Clench J, Reinberg A, Dziewanowska Z, Ghata J et al. Circadian changes in the bioavailability and effects of indomethacin in healthy subjects. Eur J Clin Pharmacol 1981;20:359-69.

194. Ollagnier M, Decousus H, Cherrah Y, Levi F et al. Circadian changes in the pharmacokinetics of oral ketoprofen. Clin Pharmacokinet 1987;12:367-78.

195. Levi F, Le Louarn C, Reinberg A. Timing optimizes sustained-release indomethacin treatment of osteoarthritis. Clin Pharmacol Ther 1985;37:77-84.

196. Guissou P, Cuisinaud G, Llorca G, Lejeune E et al. Chronopharmacokinetic study of a prolonged release form of indomethacin. Eur J Clin Pharmacol 1983;24:667-70.

197. Perpoint B, Mismetti P, Simitsidis S, Hocquart J et al. Dosing time optimizes sustained-release ketoprofen treatment of osteoarthritis. Chronobiol Int 1994;11:119-25.

198. Bornschein RL,Crockett RS, Smith RP. Diurnal variations in the analgesic effectiveness of morphine in mice. Pharmacol Biochem Behav 1977:6:621-62.

199. Guney HZ, Gorgun CZ, Tunçtan B, Uludag O et al. Circadian-rhythm-dependent effects of L-NG-nitroarginine methyl ester (L-NAME) on morphine-induced analgesia. Chronobiol Int 1998;15:283-9.

200. Graves DA, Batenhorst RL, Bennett RL, Wettstein JG et al. Morphine requirements using patient-controlled analgesia: influence of diurnal variation and morbid obesity. Clin Pharm 1983;2:49-53.

201. Liu XP, Song JG. [Chronopharmacology of tramadol in mice]. Yao Xue Xue Bao 2001;36:561-4.

202. Hummel T, Kraetsch HG, Lötsch J, Hepper M et al. Analgesic effects of dihydrocodeine and tramadol when administered either in the morning or evening. Chronobiol Int1995;12:62-72.

203. Anastasopoulou-Sampani D, Sampanis E, Karargiris G. The need for analgesia in elective cholecystectomies influenced by the time of day the operation is performed. Acta Anaesthesiol Scand 1996;40:955.

204. Ritschel WA, Bykadi G, Ford DJ, Bloomfield SS et al. Pilot study on disposition and pain relief after i.m. administration of meperidine during the day or night. Int J Clin Pharmacol Ther Toxicol 1983;21:218-23.

205. Oliverio A, Castellano C, Puglisi-Allegra S. Opiate analgesia: evidence for a circadian rhythm in mice. Brain Res 1982;249:265-270.

206. Wesche DL, Frederickson RC. The role of the pituitary in the diurnal variation in tolerance to painful stimuli and brain enkephalin levels. Life Sci 1981;29:2199-205.

207. Kerdelhue B, Karteszi M, Pasqualini C, Reinberg A et al. Circadian variations in beta-endorphin concentrations in pituitary and in some brain nuclei of the adult male rat. Brain Res 1983;261:243-8.

208. Puglisi-Allegra S, Castellano C, Oliverio A. Circadian variations in stress-induced analgesia. Brain Res 1982;252:373-6.

209. Naber D, Cohen RM, Pickar D, Kahn NH et al. Episodic secretion of opioid activity in human plasma and monkey CSF. Life Sci 1981a;28:931-5.

210. Naber D, Wirz-Justice A, Kafka MS. Circadian rhythm in rat brain opiate receptor. Neurosci Lett 1981;21:45-50.

211. Labrecque G, Lepage-Savary D, Poulin E. Time-dependent variations in morphine-induced analgesia. Annu Rev Chronopharmacol 1988;5:135-8.

212. Auvil-Novak SE, Novak RD, Smolensky MH, Kavanagh JJ et al. Twenty-four hour variation in self-administration of morphine sulfate and hydromorphone by postsurgical gynecologic cancer patient. Annu Rev Chronopharmacol 1988;5:343-6.

213. Vanier MC, Labrecque Cl, Lepage-Savary D. Temporal changes in the hydromorphone analgesia in cancer patients. In: Smolensky MH, Labrecque G, Reinberg A, Lemmer B et al. editors. Proceedings of the 5th International Conference on Biological Rhythms and Medications. Houston: Pergamon press, 1992. Abstr. XIII-8.

214. Pan Peter HMD, Lee Sherman MD, Harris Lynne BSN. Chronobiology of Subarachnoid fentanyl for Labor Analgesia. Anesthesiol 2005;103:595-9.

215. Fischer U, Freyse EJ, Albrecht G, Gebel G et al. Daily glucose and insulin rhythms in diabetic dogs on the artificial beta cell. Exp Clin Endocrinol 1985;85:27-37.

216. De Souza CJ, Meier AH. Circadian and seasonal variations of plasma insulin and cortisol concentrations in the Syrian hamster, Mesocricetusauratus. Chronobiol Int 1987;4:141-51.

217. Peschke E, Peschke D. Evidence for a circadian rhythm of insulin release from perfused rat pancreatic islets. Diabetologia 1998;41:1085-92.

218. Pauly JE, Scheving LE. Circadian rhythms in blood glucose and the effect of different lighting schedules, hypophysectomy, adrenal medullectomy and starvation. Am J Anat 1967;120:627-36.

219. Jolin T, Montes A. Daily Rhythm of Plasma Glucose and Insulin Levels in Rats. Horm Res 1973;4:153-6.

220. Louis-Sylvestre J. Feeding and metabolic patterns in rats with truncular vagotomy or with transplanted beta-cells. Am J Physiol 1978;235:119-25.

221. Bizot-Espiard JG, Double A, Guardiola-Lemaitre B, Delagrange P et al. Diurnal rhythms in plasma glucose, insulin, growth hormone and melatonin levels in fasted and hyperglycemic rats. Diabetes Metab 1998;24:235-40.

222. Delatre E, Cipolla-Neto J, Boschero AC. Diurnal variations in insulin secretion and K^+ permeability in isolated rat islets. Clin and Exp Pharmacol Physiol 1999;26:505-10.

223. Hansen AP, Johansen K. Diurnal patterns of blood glucose, serum free fatty acids, insulin, glucagon and growth hormone in normal and juvenile diabetics. Diabetologia 1970;6:27-33.

224. Volker M, Achim P, Kerstin MO, Werner K et al. Preserved circadian rhythm of serum insulin concentration at low plasma glucose during fasting in lean and overweight humans. Metabolism 2004;53:1449-53.

225. Whichelow MJ, Sturge RA, Keen H, Jarret RJ et al. Diurnal variation in response to intravenous glucose. Br Med J 1974;1:488-91.

226. Penicaud L, Le Magnen J. Aspects of the neuroendocrine bases of the diurnal metabolic cycle in rats. Neurosci Biobehav Rev 1980;4:39-42.

227. Shapiro ET, Tillil H, Polonsky KS et al. Oscillations in insulin secretion during constant glucose infusion in normal man: relationship to changes in plasma glucose. J Clin Endocrinol Metab 1988;67:307-14.

228. Polonsky KS, Given BD, Van Cauter E. Twenty-four-hour profiles and pulsatile patterns of insulin secretion in normal and obese subjects. J Clin Invest 1988;81:442-8.

229. Fudala PJ, Johnson RE. Development of opioid formulations with limited diversion and abuse potential. Drug Alcohol Depend 2006;83:S40-7.

230. Hoskin PJ, Hanks GW. Opioid agonist-antagonist drugs in acute and chronic pain states. Drugs 1991;41:326-44.

231. Bidlack JM, McLaughlin JP, Wentland MP. Partial opioids. Medications for the treatment of pain and drug abuse. Ann N Y Acad Sci 2000;909:1-11.

232. Suzuki T, Narita M, Misawa M, Nagase H. Pentazocine-induced biphasic analgesia in mice. Life Sci 1991;48:1827-35.

233. Veena V, Mediratta PK, Sharma KK. Interaction of pentazocine with calcium channel blocking drugs during chemical and thermal pain in mice. Indian J Physiol Pharmacol 2000;44:255-64.

234. Shu H, Hayashida M, Arita H, Huang W et al. Pentazocine induced antinociception is mediated mainly by μ-opioid receptors and compromised by κ-opioid receptors in mice. J Pharmacol Exp Ther 2011;338:579-87.

235. Gerhard Vogel H. Drug discovery and evaluation: pharmacological assays. 2nd ed. Germany: Springer-Verlag; 2002.

236. Koster R, Anderson M, DeeBeer AJ. Acetic acid for analgesic screening. Fed Proc 1959;18:412-16.

237. Tjølsen A, Berge OG, Hunskaar S, Rosland JH et al. The formalin test: an evaluation of the method. Pain 1992;51:5-17.

238. Tjølsen A, Hole K. Animal Models of Analgesia. In: Dickensen A, Besson J, editors. The Pharmacology of pain. Berlin: Springer Verlag; 1997.

239. Grossman ML, Basbaum AI, Fields HL. Afferent and efferent connections of the rat tail flick reflex (a model used to analyze pain control mechanisms). J Comp Neurol 1982;206:9-16.

240. Eddy NB, Leimbach D. Synthetic analgesics. II. Dithienylbutenyl and dithienylbutylamines. J Pharmacol Exp Ther 1953;107:385-93.

241. Thangavel P, Sumathiral K, Karthikeyan S, Ramaswamy M. Endocrine response of the fresh water teleost, Sarotherodon mossambicus (Peters) to dimecron exposure. Chemosphere 2005;61:1083-92.

242. Parasuraman S Raveendran R and Kesavan R. Blood sample collection in small laboratory animals. J Pharmacol Pharmacother 2010;1:87-3.

Table 1

Relation between serum insulin, blood glucose and pain threshold in control, pentazocine (4mg/kg; i.p), naloxone high dose pretreatment (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) in acetic acid induced abdominal constrictions in mice.

Treatment mg/kg; i.p	Serum insulin (µIU/mL)	Number of abdominal constrictions	Blood glucose (%)
Control	13.20±0.15	32.0 ± 0.73	106.49 ± 3.47
Pentazocine 4	15.83 ± 0.30[a]	15.33 ± 0.33[a]	103.99 ± 3.50 [ns]
Naloxone pretreatment 5	11.33 ± 0.34[a]	31.66 ± 0.84 [ns]	104.38 ± 3.03[ns]
Naloxone pretreatment 0.005	17.55±0.20 [a, b]	11.83 ± 0.48 [a, b]	102.88 ± 3.74[ns]

Each value represents mean ± SEM of six observations.

[a]P<0.01 compared with control.

[b]P<0.01 compared with pentazocine treatment.

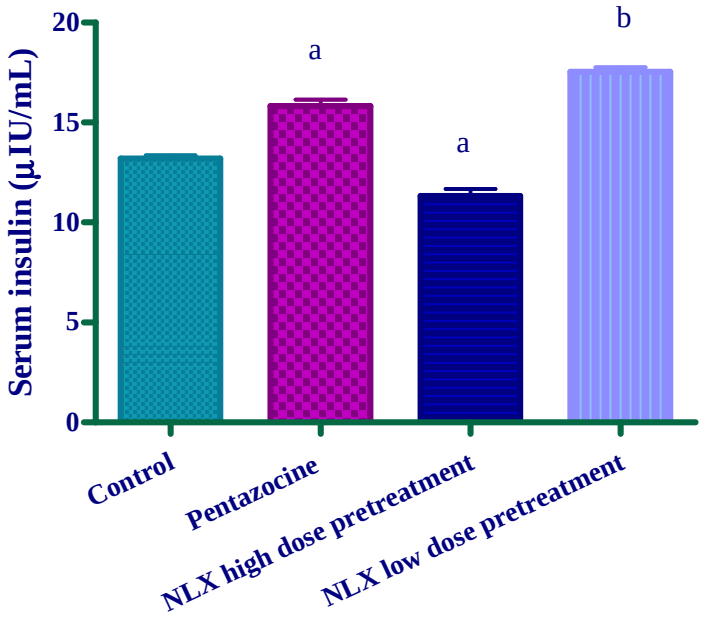


Figure 6

Effect of pentazocine (4mg/kg; i.p), naloxone high dose pretreatment (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) on serum insulin in acetic acid induced abdominal constrictions method in mice. (NLX: naloxone). n=6; values are expressed as mean ± SEM; (One way ANOVA followed by Dunnett's't' test). [a]P<0.01 compared with control. [b]P<0.01 compared with pentazocine treatment

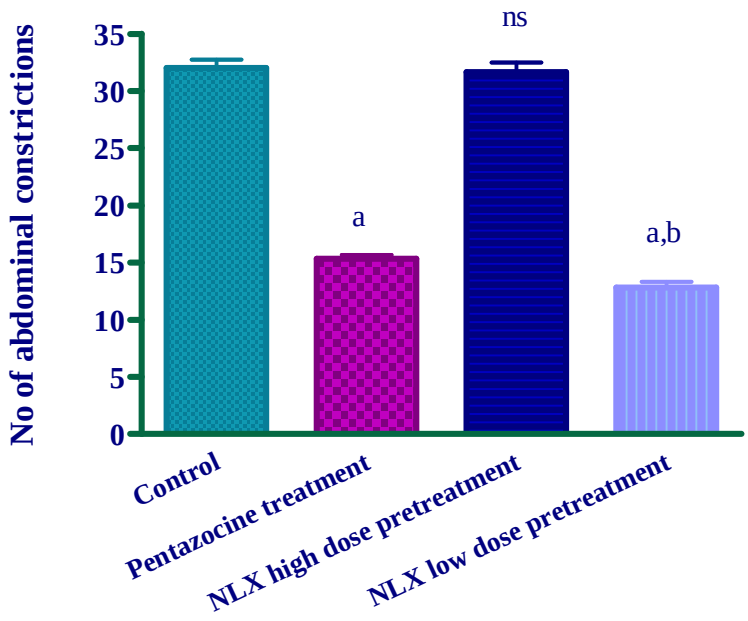


Figure 7

Effect of pentazocine (4mg/kg; i.p), naloxone high dose pretreatment (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) on number of abdominal constrictions in acetic acid induced abdominal constrictions method in mice. (NLX: naloxone). n=6; values are expressed as mean ± SEM; (One way ANOVA followed by Dunnett's't' test). [a]P<0.01 compared with control. [b]P<0.01 compared with pentazocine treatment.

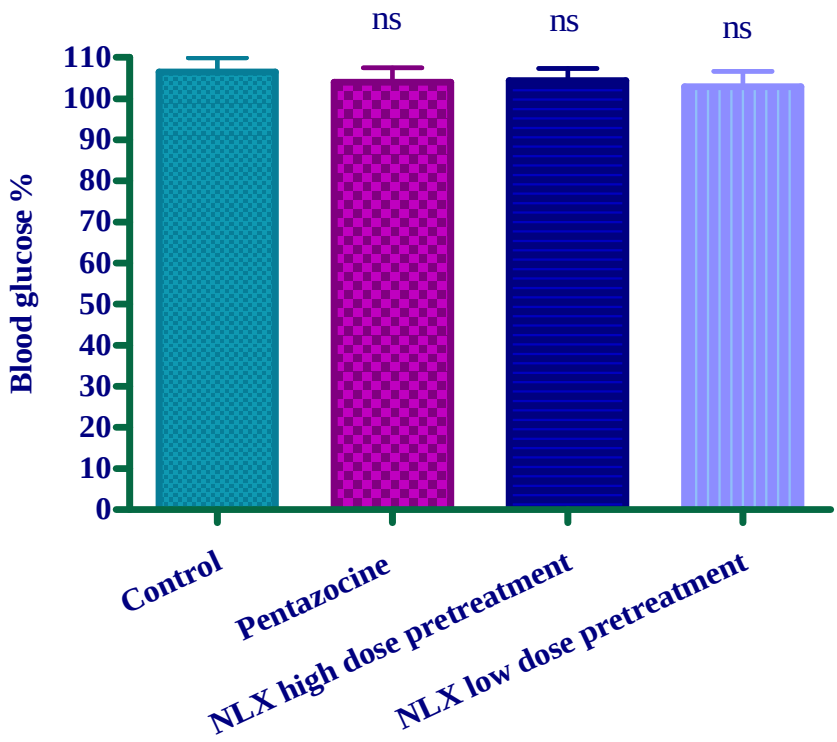


Figure 8

Effect of pentazocine (4mg/kg; i.p), naloxone high dose pretreatment (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) on blood glucose in acetic acid induced abdominal constrictions method in mice. (NLX: naloxone). n=6; values are expressed as mean ± SEM; (One way ANOVA followed by Dunnett's't' test). ns: not significant.

Table 2

Relation between serum insulin, blood glucose and pain threshold in control, pentazocine (30mg/kg; i.p), naloxone high dose pretreatment (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) in formalin induced nociception in mice (chronic phase).

Treatment mg/kg; i.p	Serum insulin (µIU/mL)	Paw licking response time in seconds	Blood glucose (%)
Control	14.11±0.20	87.5±0.43	110.61±5.01
Pentazocine 30	19.15±0.27[a]	52.83±0.65[a]	108.38 ±5.18 [ns]
Naloxone pretreatment 5	13.63±0.35[ns]	86.83±1.01 [ns]	109.37±4.69[ns]
Naloxone pretreatment 0.005	20.46±0.26 [a,b]	44.50±0.56[a,b]	105.45±4.41[ns]

Each value represents mean ± SEM of six observations.

[a]P<0.01 compared with control.

[b]P<0.01 compared with pentazocine treatment.

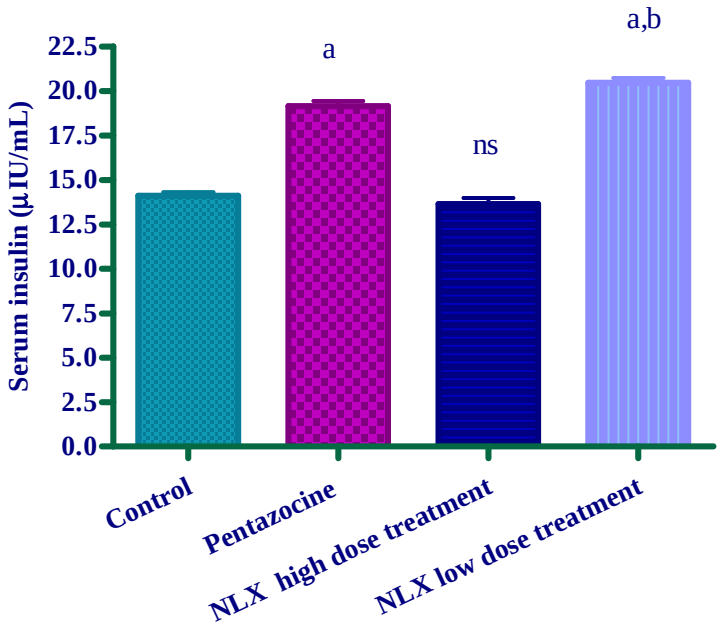


Figure 9

Effect of pentazocine (30 mg/kg; i.p), naloxone high dose pretreatment (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) on serum insulin in formalin induced nociception method. (NLX: naloxone). n=6; values are expressed as mean ± SEM; (One way ANOVA followed by Dunnett's't' test). [a]P<0.01 compared with control. [b]P<0.01 compared with pentazocine treatment.

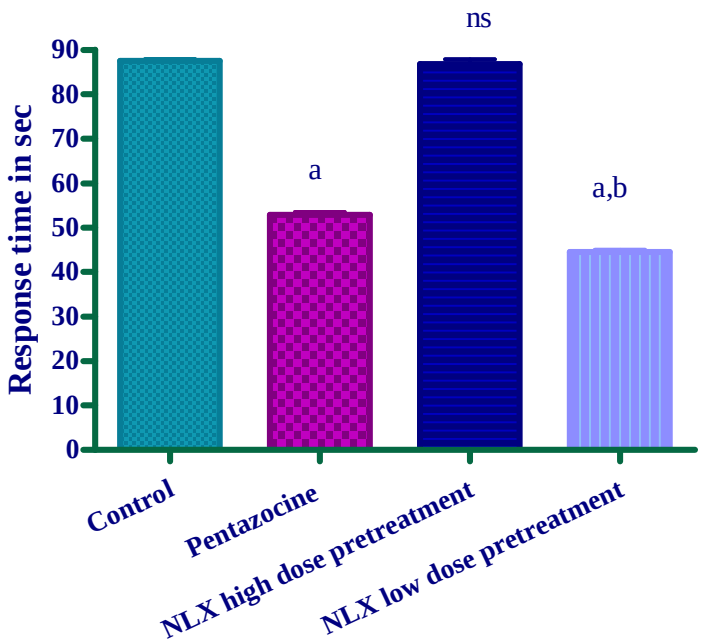


Figure 10

Effect of pentazocine (30mg/kg; i.p), naloxone high dose pretreatment (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) on response time in sec formalin induced nociception method in mice (NLX: naloxone). n=6; values are expressed as mean ± SEM; (One way ANOVA followed by Dunnett's't' test). [a]P<0.01 compared with control. [b]P<0.01 compared with pentazocine treatment.

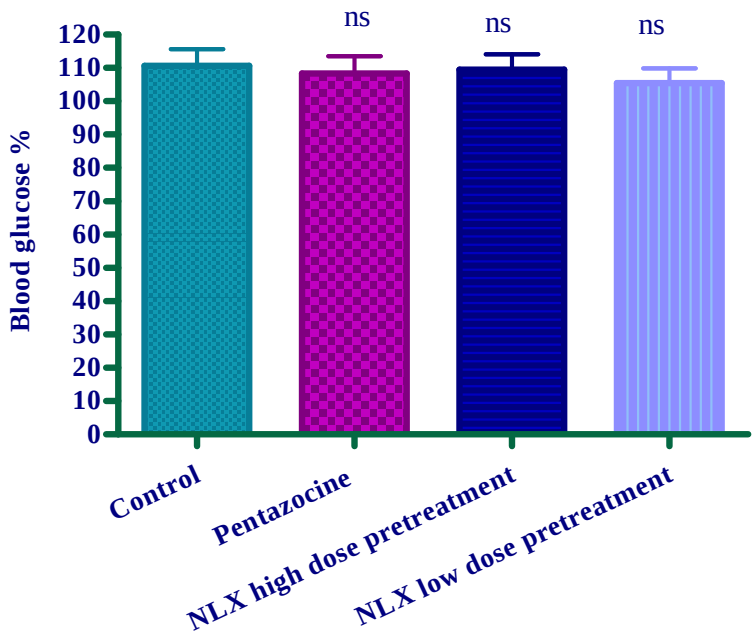


Figure 11

Effect of pentazocine (30 mg/kg; i.p), naloxone high dose pretreatment (5mg/kg; i.p.), naloxone low dose pretreatment (0.005 mg/kg; i.p.) on blood glucose level in formalin induced nociception method (NLX: naloxone). n=6; values are expressed as mean ± SEM; (One way ANOVA followed by Dunnett's't' test). ns : not significant.

Table 3

Relation between serum insulin, blood glucose and pain threshold in control, naloxone high dose pretreatment (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) in tail flick model in mice.

Treatment mg/kg; i.p	Serum insulin (µIU/mL)	Latency time	Blood glucose
Control	14.23±0.12	2.15 ± 0.11	109.87 ± 3.66
Pentazocine 30	20.58 ± 0.29[a]	3.48± 0.11[a]	114.7 ± 4.29 [ns]
Naloxone pretreatment 5	14.18 ± 0.31[a]	1.93 ± 0.12 [ns]	114.0± 2.92[ns]
Naloxone pretreatment 0.005	23.08±0.21 [a, b]	6.53± 0.13[a, b]	117.13 ± 2.87[ns]

Each value represents mean ± SEM of six observations.

[a]P<0.01 compared with control.

[b]P<0.01 compared with pentazocine treatment.

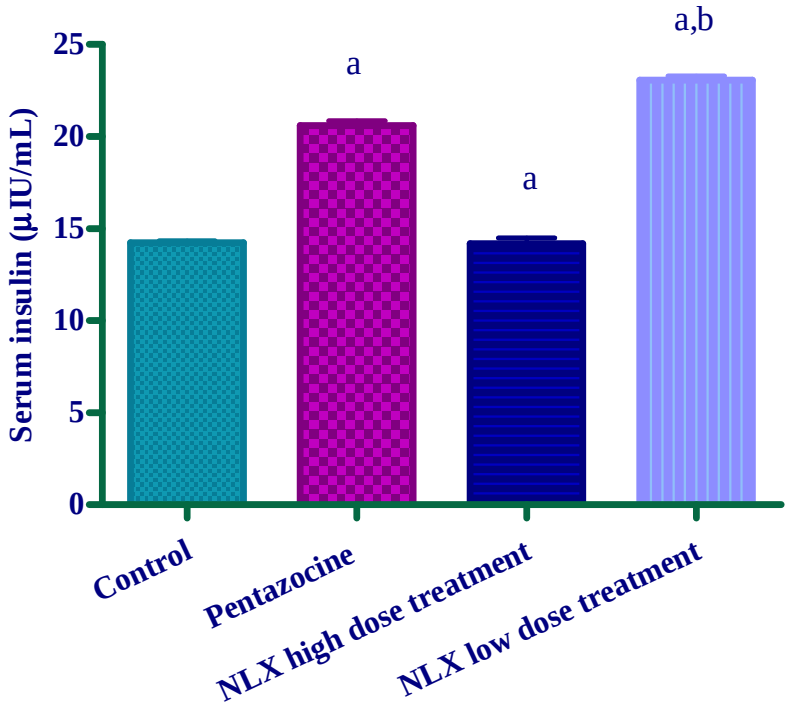


Figure 12

Effect of pentazocine (30 mg/kg; i.p), naloxone high dose pretreatment (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) on serum insulin in tail flick method (NLX; naloxone). n=6; values are expressed as mean ± SEM; (One way ANOVA followed by Dunnett's't' test). [a]P<0.01 compared with control. [b]P<0.01 compared with pentazocine treatment.

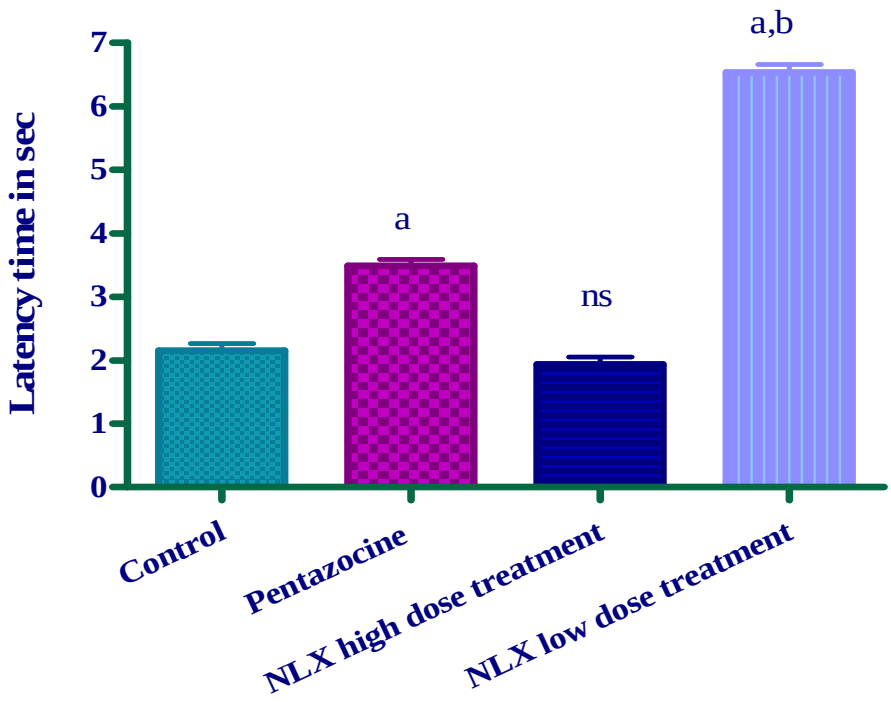


Figure 13

Effect of pentazocine (30 mg/kg; i.p), naloxone high dose pretreatment (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) on latency time in tail flick method (NLX: naloxone). n=6; values are expressed as mean ± SEM; (One way ANOVA followed by Dunnett's't' test). [a]P<0.01 compared with control. [b]P<0.01 compared with pentazocine treatment.

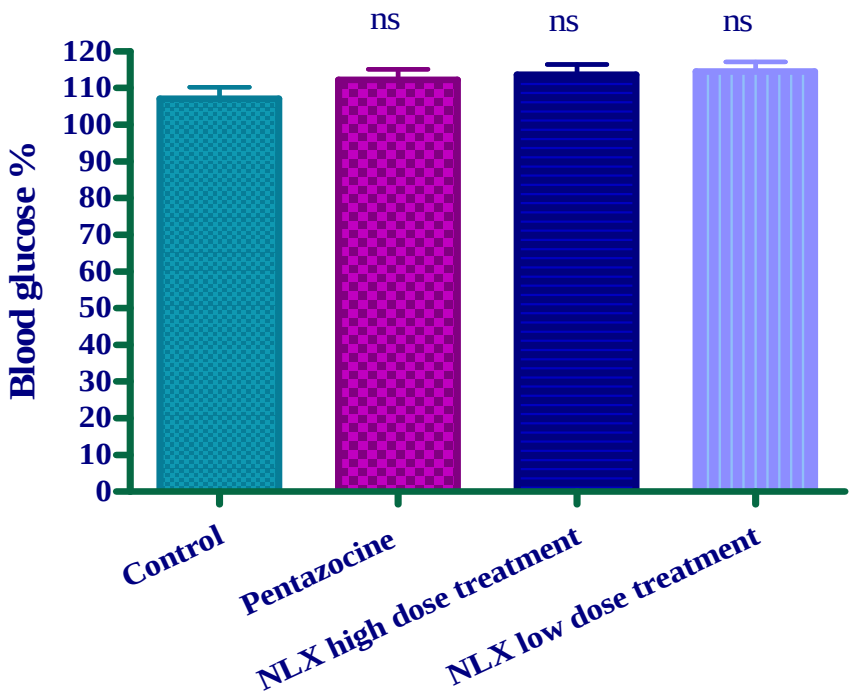


Figure 14

Effect of pentazocine (30 mg/kg; i.p), naloxone high dose pretreatment (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) on blood glucose in tail flick method (NLX : naloxone). n=6; values are expressed as mean ± SEM; (One way ANOVA followed by Dunnett's't' test).ns: not significant.

Table 4

The relation between serum insulin, blood glucose and pain threshold in control, naloxone high dose pretreatment (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) in hot plate model in mice.

Treatment mg/kg; i.p	Serum insulin (µIU/mL)	Latency time in sec	Blood glucose %
Control	15.20±0.34	5.16 ± 0.31	130.0 ± 4.33
Pentazocine 4	19.13 ± 0.70[a]	7.50± 0.67[a]	105.0± 3.88[a]
Naloxone pretreatment 5	15.52 ± 0.22[a]	5.55 ± 0.22 [ns]	129.08± 4.60[ns,a]
Naloxone pretreatment 0.005	21.40±0.41 [a, b]	9.50± 0.22[a, b]	103.85 ±3.70[a,ns]

Each value represents mean ± SEM of six observations.

[a]P<0.01 compared with control.

[b]P<0.01 compared with pentazocine treatment.

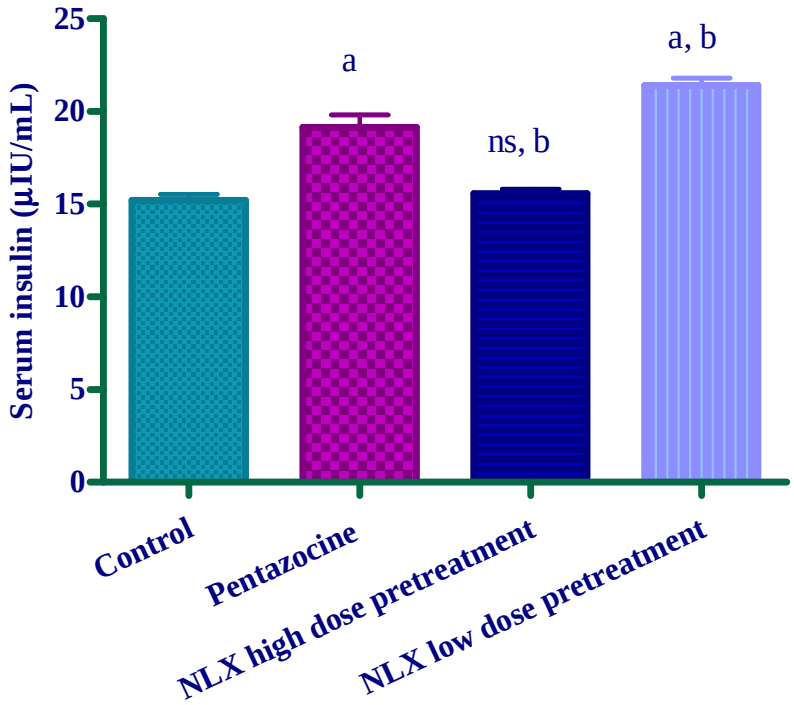


Figure 15

Effect of pentazocine (30 mg/kg; i.p), NLX high dose: naloxone high dose pretreatment (5mg/kg; i.p.) and NLX low dose: naloxone low dose pretreatment (0.005 mg/kg; i.p.) on serum insulin in hot plate method n=6; values are expressed as mean ± SEM; (One way ANOVA followed by Dunnett's't' test). [a]P<0.01 compared with control. [b]P<0.01 compared with pentazocine treatment.

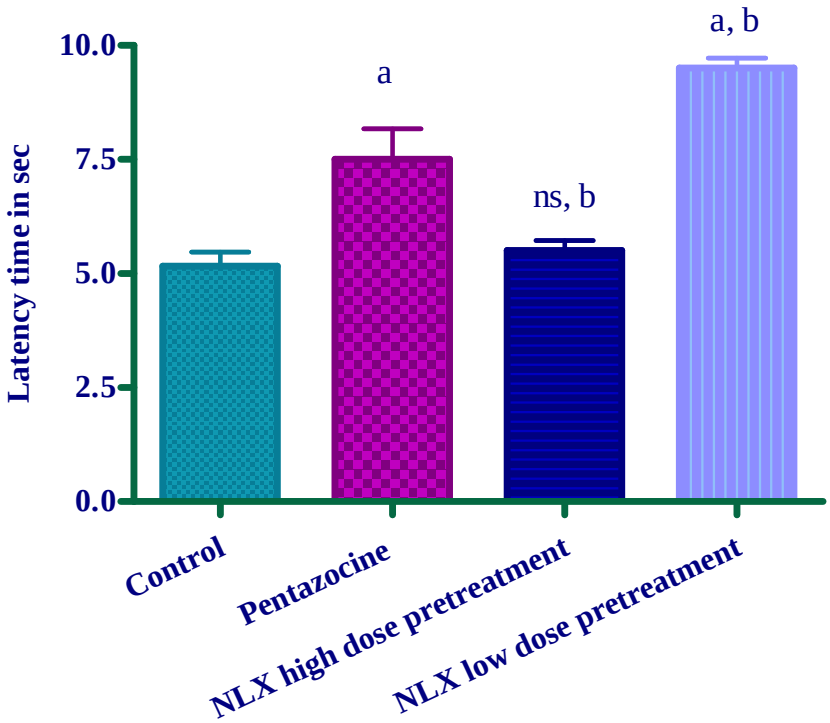


Figure 16

Effect of pentazocine (30 mg/kg; i.p), naloxone high dose pretreatment (5mg/kg; i.p.) naloxone low dose pretreatment (0.005 mg/kg; i.p.) on latency time in hot plate method (NLX: naloxone). n=6; values are expressed as mean ± SEM; (One way ANOVA followed by Dunnett's't' test). [a]P<0.01 compared with control. [b]P<0.01 compared with pentazocine treatment.

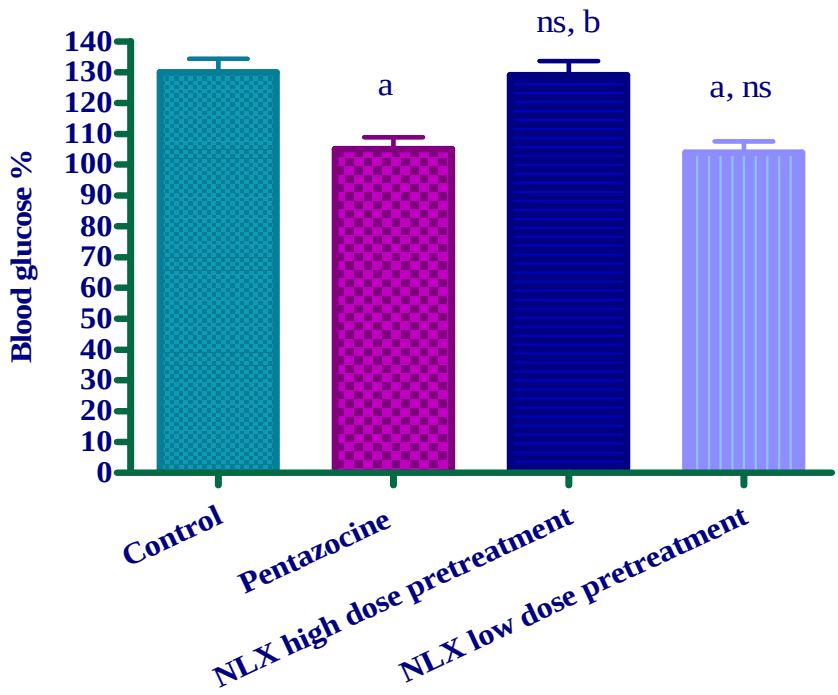


Figure 17

Effect of pentazocine (30 mg/kg; i.p), naloxone high dose pretreatment (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) on blood glucose in hot plate method (NLX: naloxone). n=6; values are expressed as mean ± SEM; (One way ANOVA followed by Dunnett's't' test). [a]P<0.01 compared with control. [b]P<0.01 compared with pentazocine treatment.

Table 5
Effect of diurnal rhythm on reaction time, endogenous serum insulin level and blood glucose in control, pentazocine (4mg/kg; i.p.), naloxone high dose pretreatment (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) in acetic acid induced abdominal constrictions model. (n=6)

Time	Reaction time(sec) (Mean±SEM)				Serum insulin level (µIU/mL) (Mean±SEM)				Blood glucose(%) (Mean±SEM)			
	Control	Pentazocine	Naloxone high dose	Naloxone low dose	Control	Pentazocine	Naloxone high dose	Naloxone low dose	Control	Pentazocine	Naloxone high dose	Naloxone low dose
12.00 h (light cycle)	32.0 ±0.73	15.33 ± 0.33[a]	31.66 ± 0.84 [ns]	11.83 ± 0.48 [a,b]	13.20 ±0.15	15.83 ± 0.30[a]	11.33 ± 0.34[a]	17.55 ±0.20 [a, b]	106.49 ± 3.47	103.99 ± 3.50 [ns]	104.38 ± 3.03[ns]	102.88 ± 3.74[ns]
24.00 h (dark cycle)	17.66 ±0.33[c]	03.33 ± 0.33[a, c]	18.16 ± 0.87 [c]	01.33 ±0.49[a,b,c]	14.25 ±0.28[e]	17.33 ± 0.48[a,f]	13.53 ± 0.42[a,d]	18.80 ±0.23[a,b,d]	105.85 ± 3.47	106.31 ± 2.42[ns]	107.08 ± 3.66 [ns]	101.68 ± 4.12 [ns]

Each value represents mean ± SEM of six observations.

[a]P<0.01 compared with control.
[b]P<0.01 compared with pentazocine treatment.
[c]P<0.0001 compared with light cycle
[d]P<0.002 compared with light cycle
[e]P<0.007 compared with light cycle
[f]P<0.02 compared with light cycle

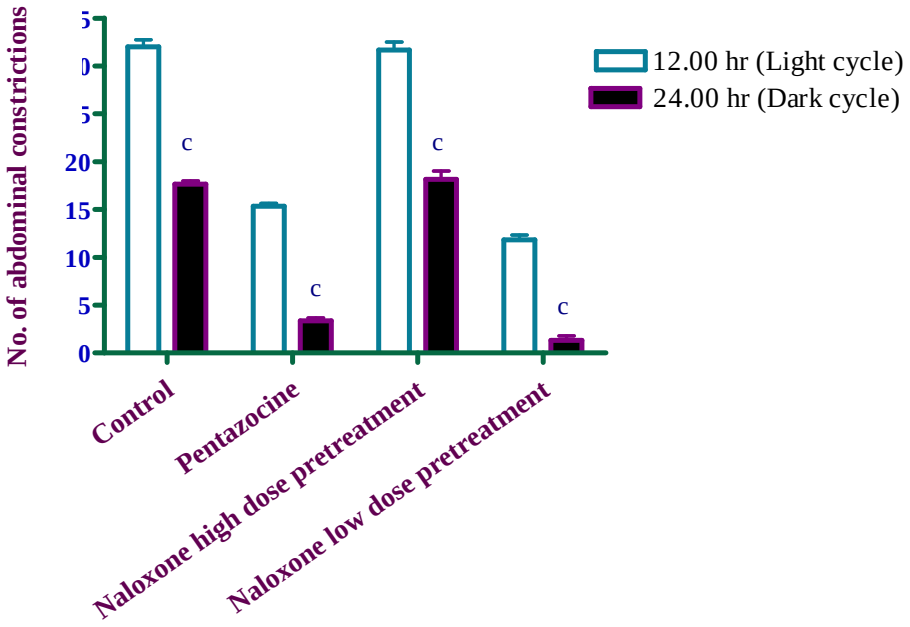


Figure 18

Effect of diurnal rhythm on reaction time in control, pentazocine (4mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p) in acetic acid induced abdominal constrictions model. n=6; the values are expressed as mean ± SEM; (Student's t test). $^{c}P<0.0001$ compared with light cycle.

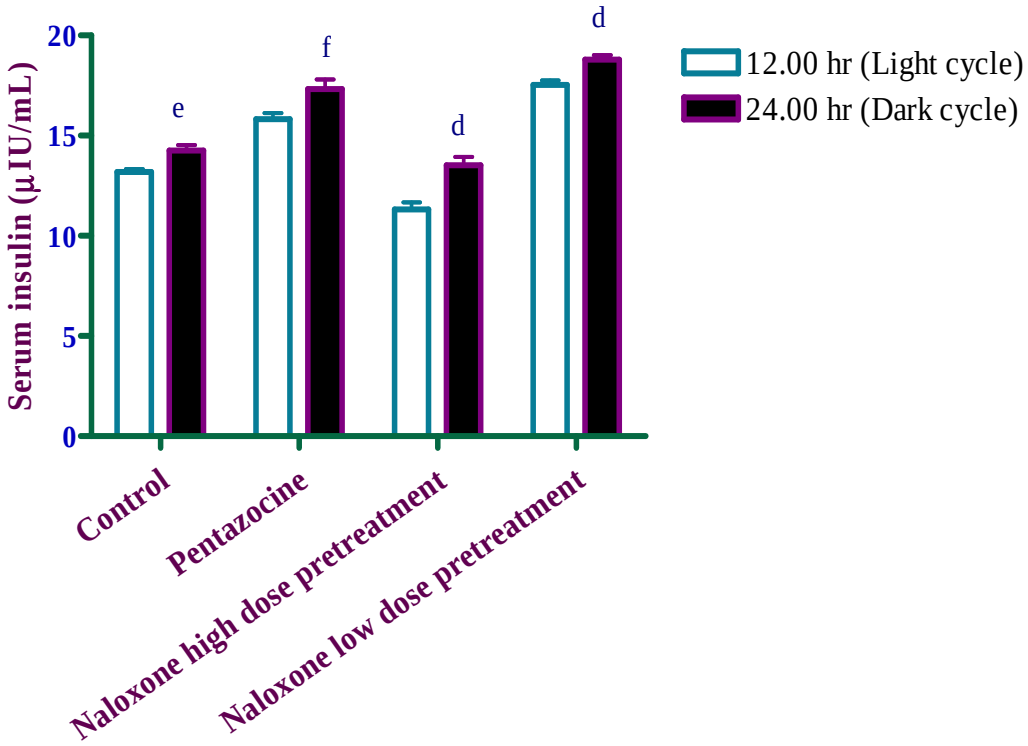


Figure 19

Effect of diurnal rhythm on serum insulinin control, pentazocine (4mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p) in acetic acid induced abdominal constrictions model. n=6; the values are expressed as mean ± SEM; (Student's t test). $^{d}P<0.002$; $^{e}P<0.007$; $^{f}P<0.02$ compared with light cycle.

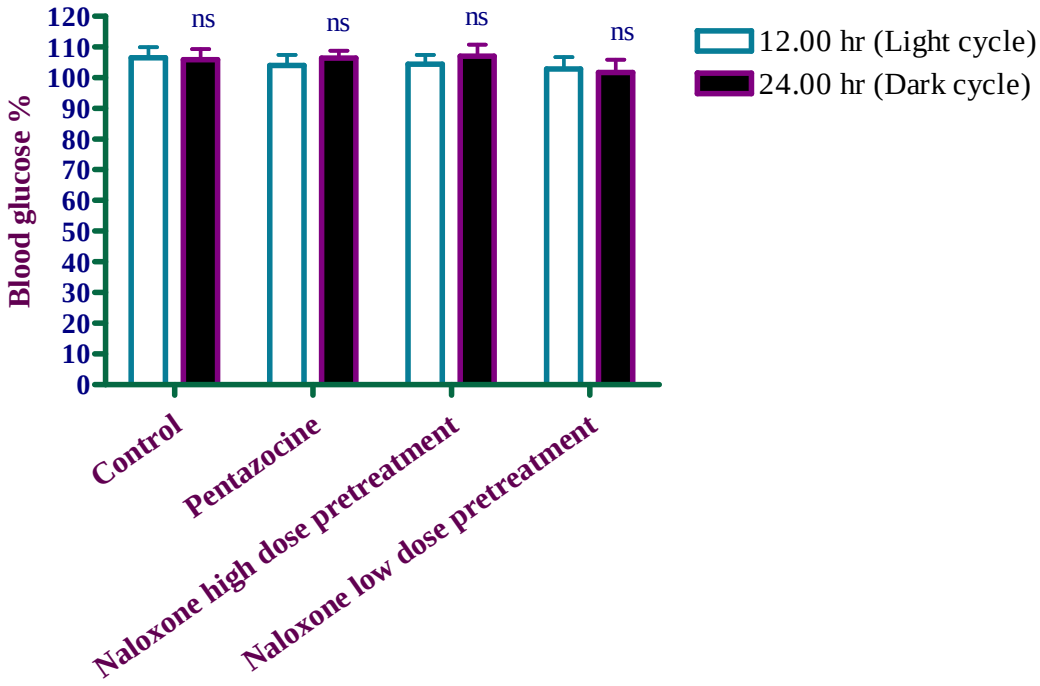


Figure 20

Effect of diurnal rhythm on blood glucosein control, pentazocine (4mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p)in acetic acid induced abdominal constrictions model. n=6; the values are expressed as mean ± SEM; (Student's t test). ns: not significant.

Table 6

Effect of diurnal rhythm on reaction time, endogenous serum insulin level and blood glucose in control, pentazocine (30mg/kg; i.p.), naloxone high dose (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) in formalin induced nociception model. (n=6)

Time	Reaction time(sec) (Mean±SEM)				Serum insulin level (µIU/mL) (Mean±SEM)				Blood glucose(%) (Mean±SEM)			
	Control	Pentazocine	Naloxone high dose	Naloxone low dose	Control	Pentazocine	Naloxone high dose	Naloxone low dose	Control	Pentazocine	Naloxone high dose	Naloxone low dose
12.00 h (light cycle)	87.5 ±0.43	52.83 ±0.65[a]	86.83 ±1.01	44.50 ±0.56[a,b]	14.11 ±0.20	19.15 ±0.27[a]	13.63 ±0.35	20.46 ±0.26 [a,b]	110.61 ±5.01	108.38 ±5.18 [ns]	109.37 ±4.69[ns]	105.45 ±4.41[ns]
24.00 h (dark cycle)	77.33 ±0.88[c]	39.16 ± 0.30[a, c]	78.33 ± 0.71 [c]	28.33 ± 0.33[a,b,c]	19.06 ±0.29 [c]	20.98 ± 0.35[a,d]	18.85 ± 0.26 [c]	23.36 ±0.34[a,b,c]	107.45 ± 5.28	105.68 ± 4.97 [ns]	107.84 ± 6.52 [ns]	105.69 ± 4.29 [ns]

Each value represents mean ± SEM of six observations.

[a]P<0.01 compared with control.
[b]P<0.01 compared with pentazocine treatment.
[c]P<0.0001 compared with light cycle
[d]P<0.009 compared with light cycle

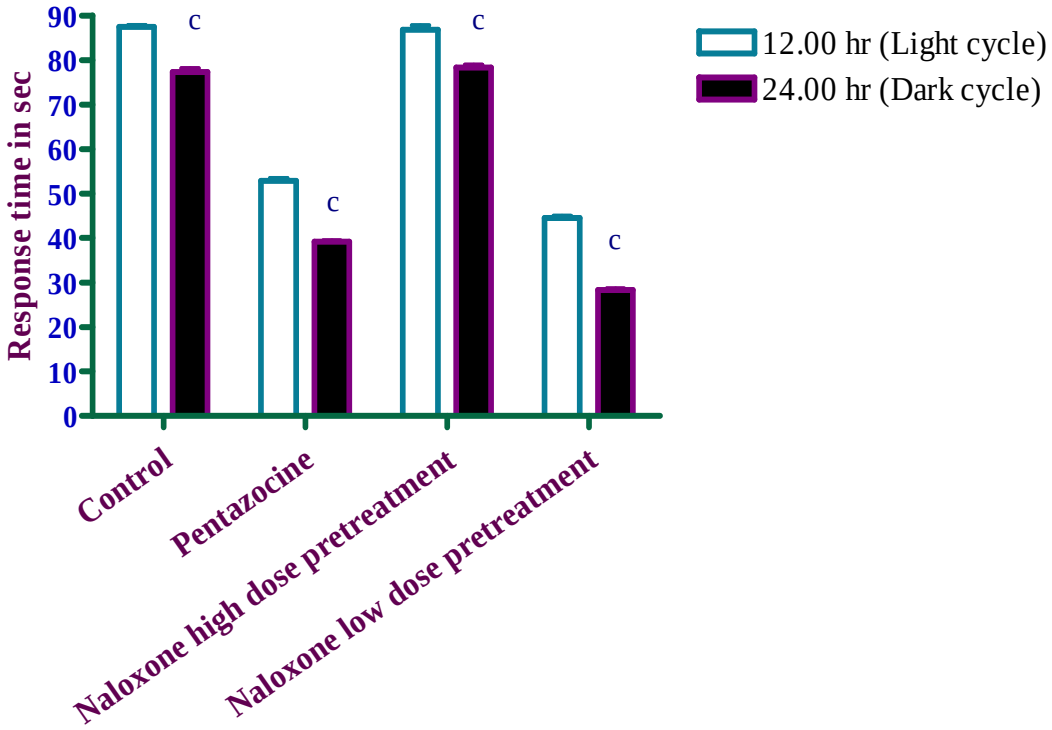


Figure 21

Effect of diurnal rhythm on response time in seconds in control, pentazocine (30mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p)in formalin induced nociception model. n=6; the values are expressed as mean ± SEM; (Student's t test). $^{c}P<0.0001$ compared with light cycle.

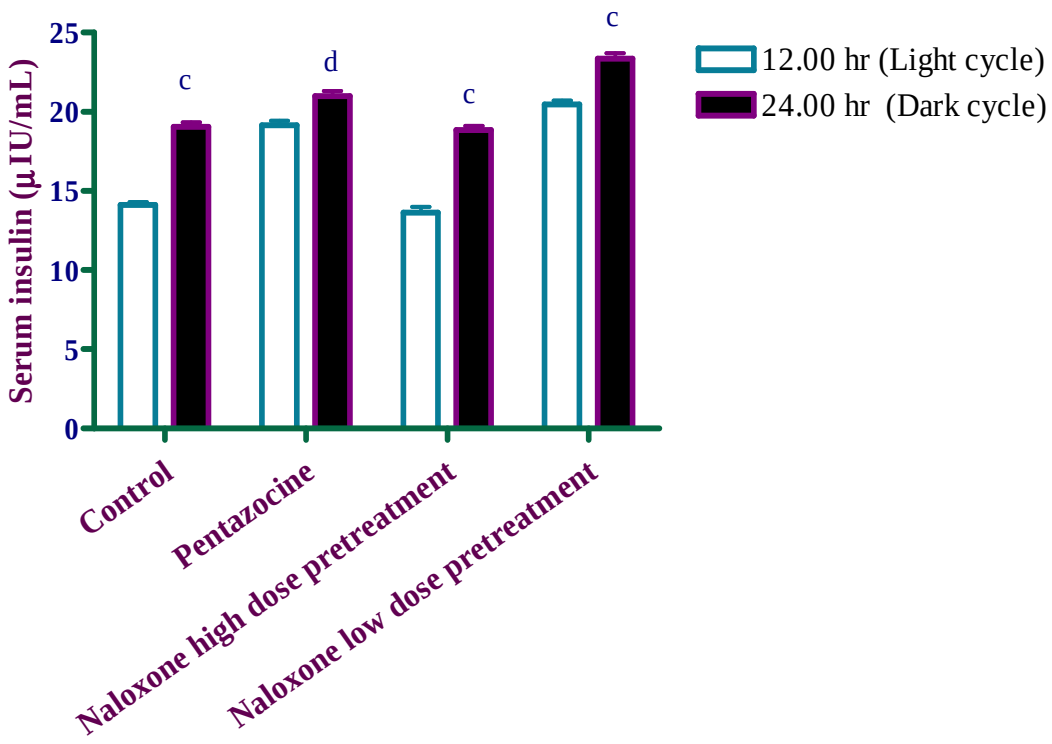


Figure 22

Effect of diurnal rhythm on serum insulinin control, pentazocine (30mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p)in formalin induced nociception model=6; the values are expressed as mean ± SEM; (Student's t test).$^{c}P<0.0001$; $^{d}P<0.009$ compared with light cycle.

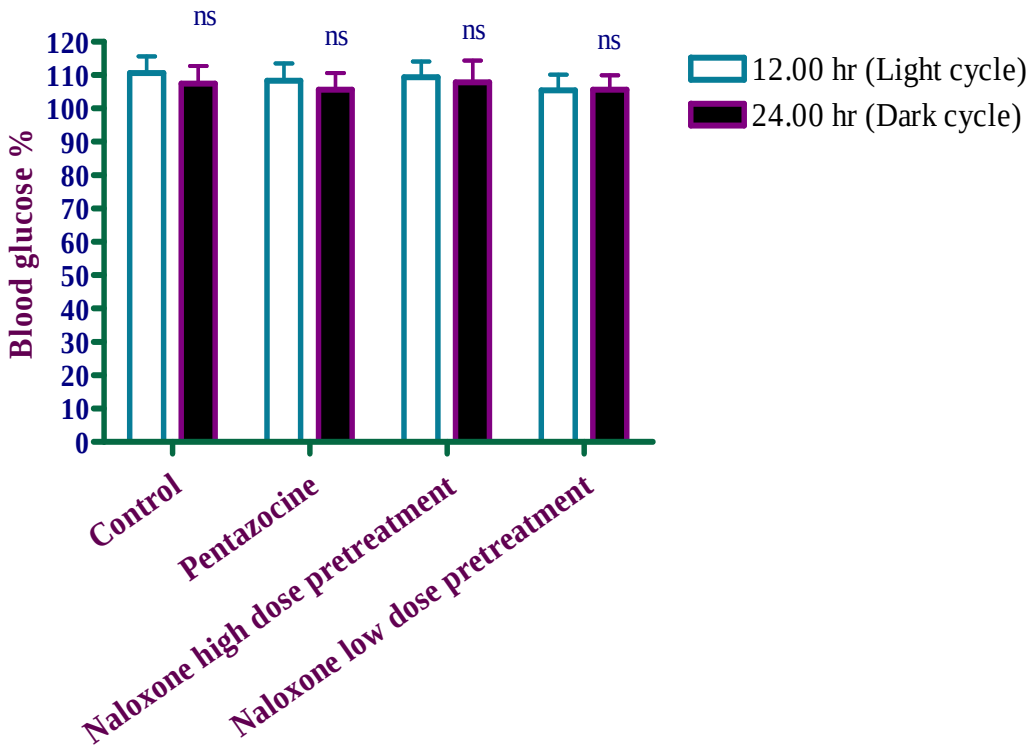


Figure 23

Effect of diurnal rhythm on blood glucose in control, pentazocine (30mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p) in formalin induced nociception model.n=6; the values are expressed as mean ± SEM; (Student's t test). ns: not significant.

Table 7

Effect of diurnal rhythm on reaction time, endogenous serum insulin level and blood glucose in control, pentazocine (30mg/kg; i.p.), naloxone high (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) in tail flick model. (n=6)

Time	Latency time(sec) (Mean±SEM)				Serum insulin level (µIU/mL) (Mean±SEM)				Blood glucose (%) (Mean±SEM)			
	Control	Pentazocine	Naloxone high dose	Naloxone low dose	Control	Pentazocine	Naloxone high dose	Naloxone low dose	Control	Pentazocine	Naloxone high dose	Naloxone low dose
12.00 h (light cycle)	2.15 ±0.11	3.48 ± 0.11[a]	1.93 ± 0.12[ns]	6.53 ± 0.13[a,b]	14.23 ±0.12	20.58 ± 0.29[a,]	14.18 ± 0.31[a]	23.08 ±0.21[a,b,]	109.87 ± 3.66	114.7 ± 4.29[ns]	114.0 ± 2.92[ns]	117.13 ± 2.87[ns]
24.00 h (dark cycle)	3.2 ±0.21[c]	05.68 ± 0.14[a, c]	2.5 ± 0.20[c]	10.83 ±0.21[a,b,c]	16.50 ±0.20[c]	22.15 ± 0.54[a,f]	15.42 ± 0.39[a,d]	24.91 ±0.33[a,b,d]	117.98 ± 4.29	114.90 ± 3.91[ns]	114.12 ± 3.28[ns]	117.13 ± 3.16[ns]

Each value represents mean ± SEM of six observations.

[a]P<0.01 compared with control.
[b]P<0.01 compared with pentazocine treatment.
[c]P<0.0001 compared with light cycle
[d]P<0.002 compared with light cycle
[e]P<0.007 compared with light cycle
[f]P<0.02 compared with light cycle

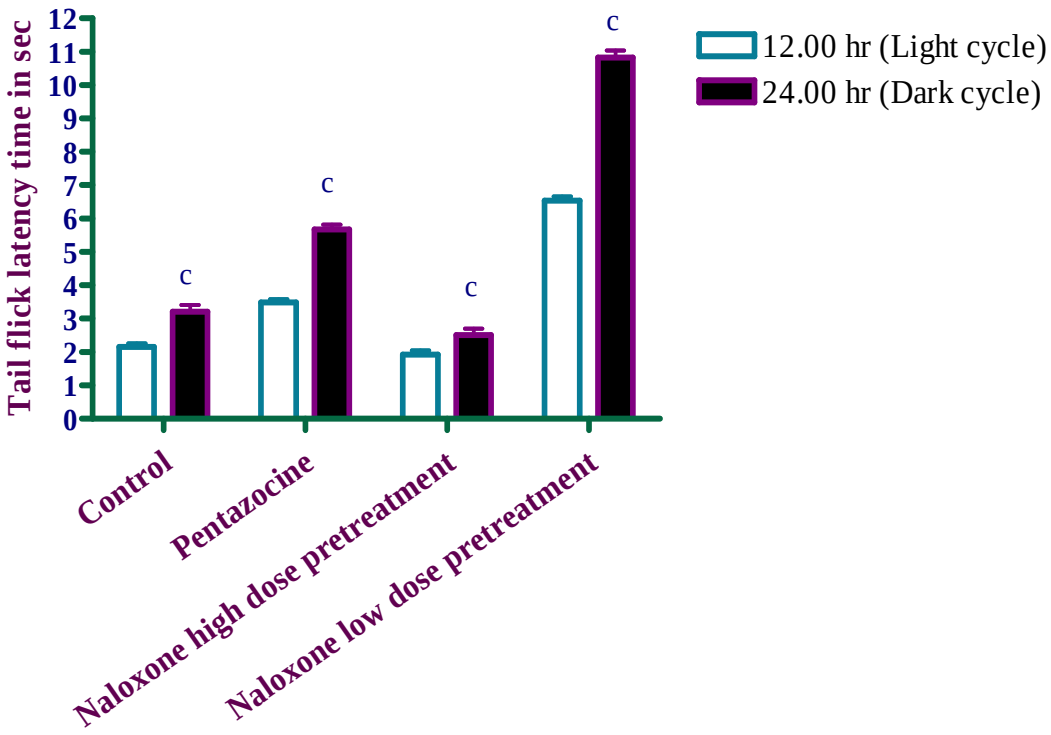


Figure 24

Effect of diurnal rhythm on tail flick latency time in sec in control, pentazocine (30mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p) in tail flick model n=6; the values are expressed as mean ± SEM; (Student's t test).^{c}P<0.0001 compared with light cycle.

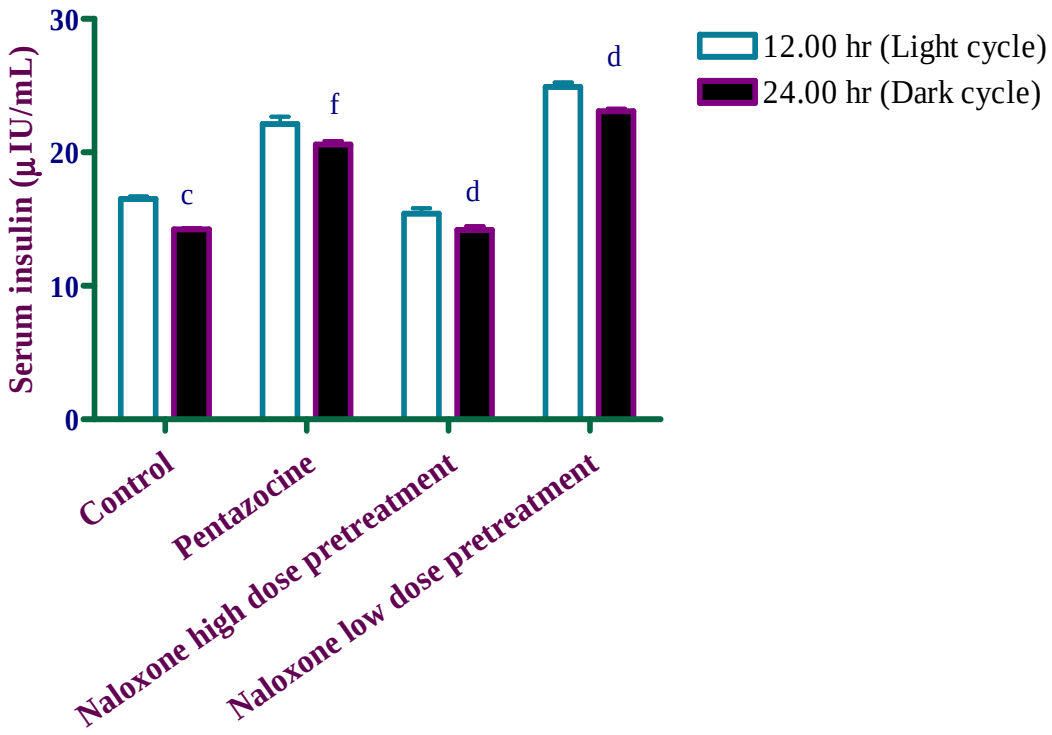


Figure 25

Effect of diurnal rhythm on serum insulin in control, pentazocine (30mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p) in tail flick model n=6; the values are expressed as mean ± SEM; (Student's t test).^{c}P<0.0001; ^{d}P<0.002; ^{f}P<0.02 compared with light cycle.

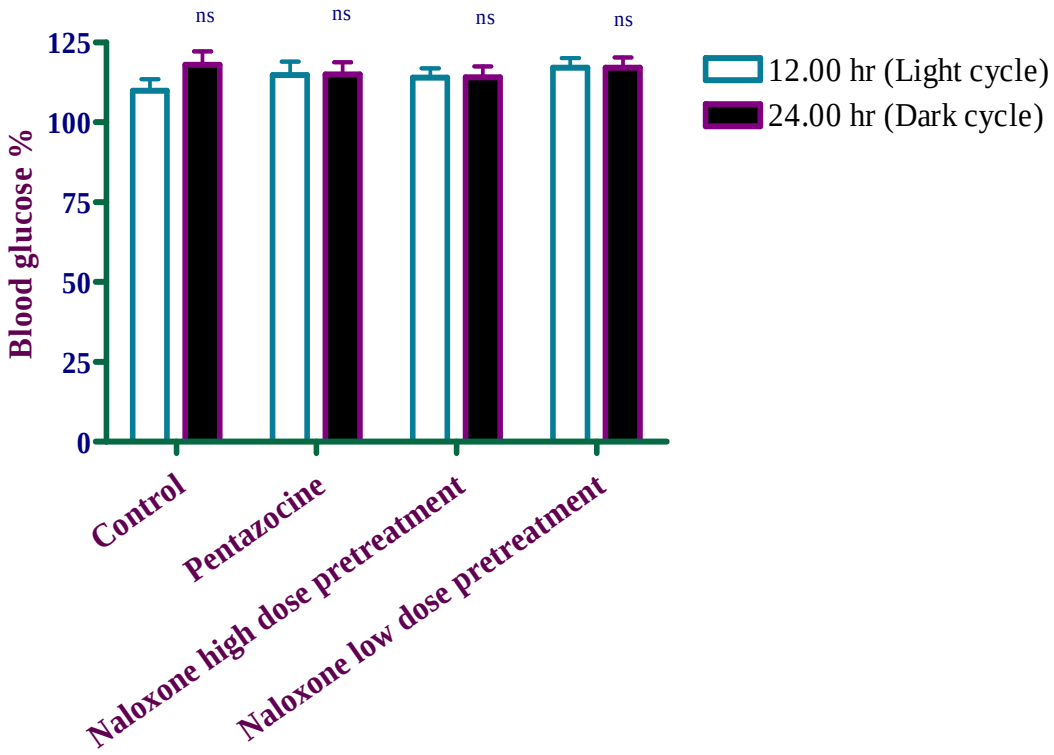


Figure 26

Effect of diurnal rhythm on blood glucose in control, pentazocine (30mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p)in tail flick model n=6; the values are expressed as mean ± SEM; (Student's t test).ns: not significant.

Table 8

Effect of diurnal rhythm on reaction time, endogenous serum insulin level and blood glucose in control, pentazocine (30mg/kg; i.p.), naloxone high dose (5mg/kg; i.p.) and naloxone low dose pretreatment (0.005 mg/kg; i.p.) in hot plate model. (n=6)

Time	Latency time(sec) (Mean±SEM)				Serum insulin level (µIU/mL) (Mean±SEM)				Blood glucose (%) (Mean±SEM)			
	Control	Pentazocine	Naloxone high dose	Naloxone low dose	Control	Pentazocine	Naloxone high dose	Naloxone low dose	Control	Pentazocine	Naloxone high dose	Naloxone low dose
12.00 h (light cycle)	5.16 ±0.31	7.50 ± 0.67[a]	5.50 ± 0.22[ns]	9.50 ±0.22[a,b]	15.20 ±0.34	19.13 ± 0.70[a]	15.52 ± 0.22[ns]	21.40 ±0.41[a,b]	130.0 ± 4.33	105.0 ± 3.88[a]	129.08 ±4.60 [ns,b]	103.85 ± 3.70[a,ns]
24.00 h (dark cycle)	8.66 ±0.21[c]	14.5 ± 0.56[a,c]	8.83 ± 0.31[ns,c]	16.67 ±0.31[a,b,c]	17.32 ±0.41[d]	21.56 ± 0.37[a,g]	17.45 ± 0.40[ns,d]	23.5 ±0.52[a,b,c]	127.97 ± 4.05	105.37 ±2.98[a]	122.18 ± 4.56[ns,b]	106.16 ± 3.25[a,ns]

Each value represents mean ± SEM of six observations.
[a]P<0.01 compared with control.
[b]P<0.01 compared with pentazocine treatment.
[c]P<0.0001 compared with light cycle
[d]P<0.002 compared with light cycle
[e]P<0.007 compared with light cycle
[f]P<0.02 compared with light cycle
[g]P<0.01 compared with light cycle

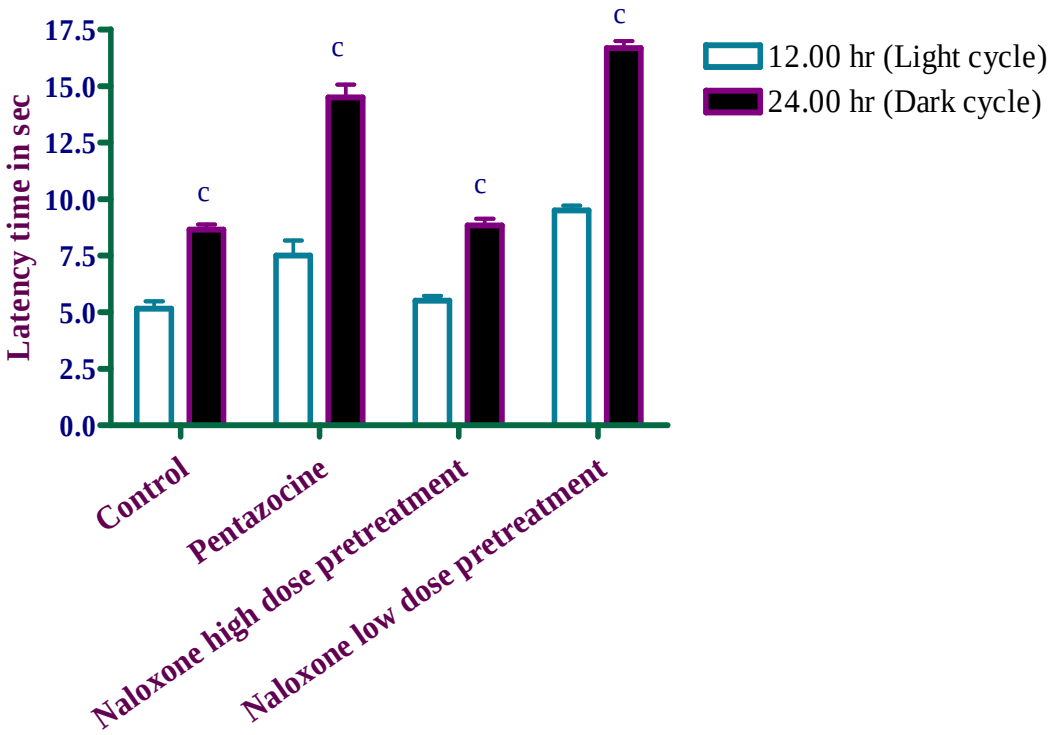


Figure 27

Effect of diurnal rhythm on latency time in control, pentazocine (30mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p)in hot plate model n=6; the values are expressed as mean ± SEM; (Student's t test). $^{c}P<0.0001$ compared with light cycle.

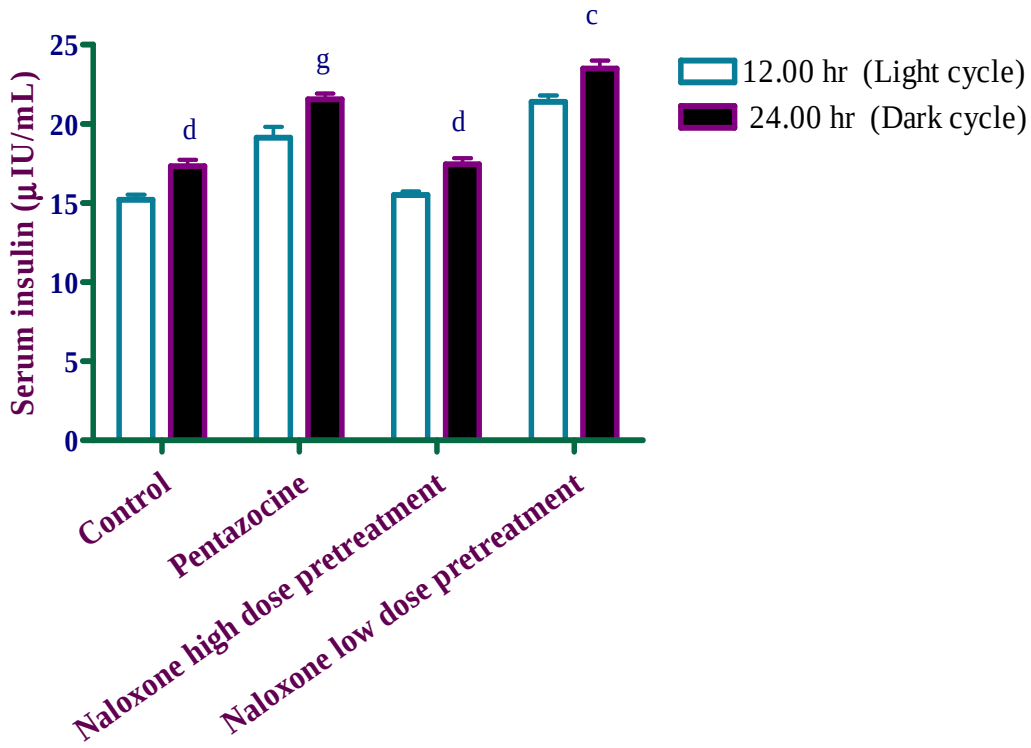


Figure 28

Effect of diurnal rhythm on serum insulin in control, pentazocine (30mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p)in hot plate model n=6; the values are expressed as mean ± SEM; (Student's t test). $^{c}P<0.0001$; $^{d}P<0.002$; $^{g}P<0.01$ compared with light cycle.

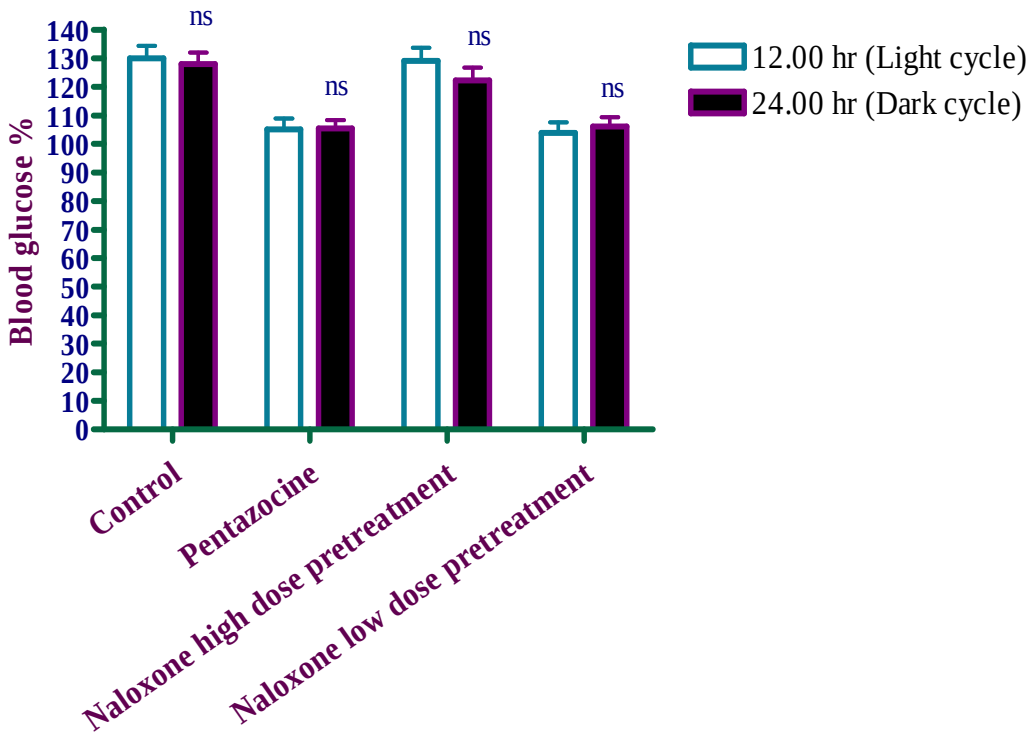


Figure 29

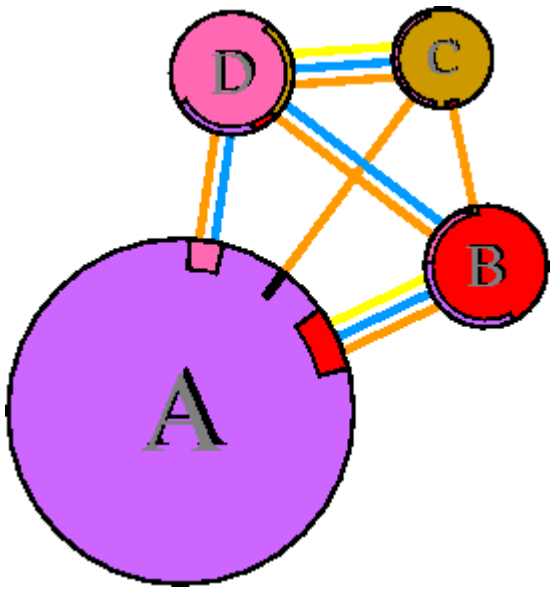
Effect of diurnal rhythm on serum insulin in control, pentazocine (30mg/kg; i.p), naloxone high dose (5mg/kg; i.p) and naloxone low dose (0.005mg/kg; i.p)in hot plate model n=6; the values are expressed as mean ± SEM; (Student's t test).ns: not significant.

Table 9

Interface statistics of interactions between protein chains.

Chains	No. of interface residues	Interface area (Å^2)	No. of salt bridges	No. of disulphide bonds	No. of hydrogen bonds	No. of non-bonded contacts
A }{ B	31 : 24	1538:1604	-	2	12	225
A }{ C	2 : 1	131:150	-	-	-	3
A }{ D	17 : 13	799:916	-	-	4	69
B }{ C	2 : 1	60:81	-	-	-	5
B }{ D	13 : 14	585:564	-	-	9	107
C }{ D	16 : 18	900:879	-	2	8	144

Fig. 30



Key: Salt bridges — Disulphide bonds — Hydrogen bonds — Non-bonded contacts

Figure.30

Schematic diagram of interactions between protein chains.

Interacting chains are joined by coloured lines, each representing a different type of interaction, as per the key above. The area of each circle is proportional to the surface area of the corresponding protein chain. The extent of the interface region on each chain is represented by a coloured wedge whose colour corresponds to the colour of the other chain and whose size signifies the interface surface area. Statistics for all the interfaces are given below.

Table 10

Interface statistics of interactions between protein chains A and B.

Chain	No. of interface residues	Interface area (Å^2)	No. of salt bridges	No. of disulphide bonds	No. of hydrogen bonds	No. of non-bonded contacts
A	31	1538	-	2	12	225
B	24	1604				

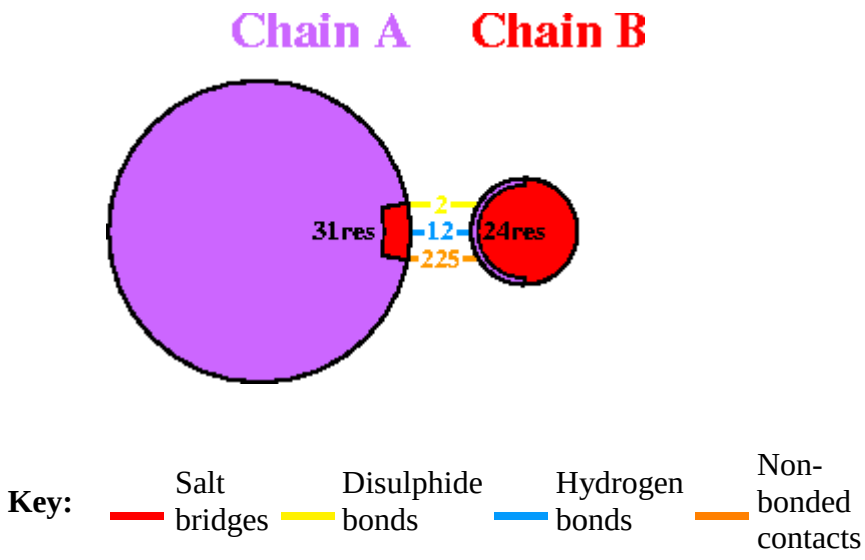


Figure.31

Schematic diagram of interactions between protein chains.

Interacting chains are joined by coloured lines, each representing a different type of interaction, as per the key above. The area of each circle is proportional to the surface area of the corresponding protein chain. The extent of the interface region on each chain is represented by the black wedge whose size signifies the interface surface area.