Chapter 6:

Discussion
DISCUSSION

The results obtained in the present study demonstrated a significant decrease in the antinociceptive effect of various analgesics, both in, diabetic and in the recipients of diabetic spleen homogenate in non-diabetic animals. Also, administration of inhibitors of cytokines, glial cell and NMDA receptor and iNOS in diabetic and SHS-recipient’s non-diabetic rats/mice, showed significant attenuation of hyperglycemia-induced decrease in analgesic effect of various analgesics and of resistance to hyperglycemia-induced thermal hyperalgesia, cold and mechanical allodynia.

It has been reported that significant degree of thermal hyperalgesia and cold and mechanical allodynia develops in rats, 3 weeks after STZ administration (Courteix et al., 1994). Therefore, rats and mice were kept for 4-6 weeks after sub-lethal dose of STZ injection, to provide sufficient time for hyperglycemia to affect pain perception. It has been previously reported that high doses of β-cell toxin STZ (200 mg/kg, i.p, once, mice) induce diabetes through a direct toxic effect on β-cells, MLDS (40 mg/kg × 5 days) in susceptible strains of mice and rats (20 mg/kg × 4 days) initiates an autoimmune destructive process similar to that observed in human type-1 DM, that allows for the study of agents potentially designed to modulate β-cell specific auto-destructive process (Elias et al., 1994; Mellado-Gil and Aguilar-Diosdado, 2004)

In this study 3 weeks diabetic rats and mice (day 28), exhibited significant thermal hyperalgesia and cold and mechanical allodynia, as compared with control rats and mice. This is in agreement with observation of Courteix et al (1994), that showed development of significant thermal hyperalgesia, cold and mechanical allodynia in STZ-treated animals. The rotarod performance time was measured, which was not affected by administration of normal saline or administration of cyclosporine, thalidomide, minocyline, pentoxifylline, Win 55, 212-2, morphine and cannabis
extract (data not shown). This indicates that test-drug-induced sedation was not involved in antiallodynic and anti-hyperalgesic effect of these drugs.

Persistent hyperglycemia is well reported to decrease the pain threshold and to markedly reduce the antinociceptive effect of morphine (Raz et al., 1988; Kamie et al., 1992), NSAIDs (Courteix et al., 1994; Tsiklauri and Tsagareli, 2006; Tsiklauri et al., 2006) cannabinoids (Matias et al., 2006; Zhang et al., 2007) and neurosteroids (Pesaresi et al, 2011). However, the mechanism of the decrease in pain threshold and antinociceptive effect of analgesics, as a consequence of diabetes, is not known.

Various mechanisms have been proposed to be involved in abnormal pain behavior and analgesics tolerance, including activation of NMDA and MAPK receptor (Adam et al., 2008; Chen et al., 2009), PKA (Smith et al., 1999), PKC (Evcimen and King, 2007; Geraldes and King, 2010), receptor desensitization (Courteix et al., 1998), an increased oxidative stress (Obrosova et al., 2007), increase in cytokines (Johnston et al., 2004) and NO levels (Grover et al., 2000; Ahmed et al., 2006). Moreover, it has been demonstrated that the inhibitory effect of morphine and cannabinoids on spinothalamic tract neurons is substantially reduced in diabetic rats, suggesting inadequate presence or dysfunction of μ-opioid and CB-1 receptors in the spinal cord dorsal horn in diabetes (Chen et al., 2002; Zhang et al., 2007).

Both cannabinoids and opioids have been reported to share several pharmacological actions, including analgesia, hypothermia, immunosuppression, drug addiction and reward (Cichewicz and Welch, 2003; Cichewicz, 2004; Welch, 2009). Accumulating line of evidence indicates that both agents are coupled to Gi/Go GTP-binding proteins that inhibit adenyl cyclase activity (Childers et al., 1992), block voltage-dependent calcium channels (Kumar et al., 2010), activate potassium channels and stimulate the
MAP kinase cascade (Deadwyler et al., 1995; Faubert and Kaminski, 2003). Also anatomical studies have shown a similar distribution of CB1 cannabinoid and mu-opioid receptors in the dorsal horn of the spinal cord (Mansour et al., 1993; Salio et al., 2002) and in several structures within the central nervous system (CNS) (Corchero et al., 2004).

Brain areas such as the caudate putamen, dorsal hippocampus, and substantia nigra are rich in both cannabinoids and opioids receptors (Herkenham et al., 1990; Mansour et al., 1993), and the co-localization of both types of receptors is possible in periaqueductal gray (PAG), raphe nuclei, central medial thalamic nuclei and the medial basal hypothalamus, that play an important role in antinociception and in neuroendocrine effects. These drugs seem to interact, on cellular and molecular level, in their analgesic effects, as demonstrated by the ability of opioids and cannabinoids antagonists to reverse cannabinoid/opioid-induced analgesia (Welch, 1993; Cichewicz, 2003; Viganò et al., 2005). Moreover, the co-administration of morphine (mu-opioid agonist) and Win 55,212-2 (CB-1-receptor agonist) enhanced the antinociceptive effect, as compared with either drug alone, in different models of acute pain (Welch and Eads, 1999; Cichewicz and McCarthy, 2003; Welch, 2009). Synergism also occured at sub-effective or sub-maximal doses of cannabinoids and opioids and these effects were blocked by cannabinoids receptor and opioids receptor antagonists (Williams et al., 2006; Cichewicz, 2004; Bushlin et al., 2010), suggesting that low doses of delta-9-tetrahydrocannabinol (THC) in conjunction with low doses of morphine could be an alternative regimen to reduce the need to escalate opioids doses, while increasing the opioids potency (Wang et al., 2005d; Paquette and Olmstead, 2005; Smith et al., 2007). Unfortunately, long term use of these drugs leads to development of tolerance and addiction (Lichtman and Martin, 2005; González et
Numerous studies indicate that chronic exposure to morphine, induces tolerance to the antinociceptive effect of THC (Thorat and Bhargava, 1994). Similarly, chronic THC, induces tolerance to the antinociceptive effect of morphine (Thorat and Bhargava, 1994; Welch, 1997).

Recently, NSAIDs have been reported to act peripherally and centrally to produces analgesic effect (Jones, 1996; Vanegas and Tortorici, 2002; Tortorici et al., 2009). A major central target of NSAIDs is the descending pain control system (Jones, 1996; Tsiklauri et al., 2008). Experimental studies have shown that this is due to an interaction of NSAIDs with endogenous opioids (Vanegas and Tortorici, 2002), along the descending pain control system (Vazquez et al., 2005; Hernandez-Delgadillo and Cruz, 2006; Ulugol et al., 2006;). In addition to this, involvement of endocannabinoids (2-AG and anandamide) in the analgesic effects of NSAIDs, have been shown at the systemic level (Gühring et al., 2002; Ulugol et al., 2006) and also locally in peripheral tissues (Ruggieri et al., 2010). It has been reported that the antinociceptive effects of NSAIDs in the spinal cord can be prevented or reversed by AM251, an antagonist/reverse agonist of the CB1 receptor (Ottani et al., 2006; Vanegas et al., 2010).

These studies indicate that bi-directional interaction exists between NSAIDs with cannabinoids and opioids in analgesic mechanism (Pernia-Andrade et al., 2004). NSAIDs act on PAG and RVM, to produce analgesia and, if repeatedly administered, induce tolerance to themselves (Tsiklauri et al. 2006; Tsiklauri et al., 2008; Tsiklauri et al., 2010) and cross-tolerance to opioids (Tortorici and Vanegas, 2000; Tortorici et al., 2004 & 2009; Tsiklauri et al., 2006). Moreover, functional reduction of opioids receptors (mu), and cannabinoids–CB-1 receptor at spinal and supra spinal neurone has been reported in diabetic patients (Chen et al., 2002; Zhang et al., 2007).
In the present study, it has been demonstrated for the first time, that Win 55,212-2 (CB-agonist) induced antinociceptive effect was significantly reduced in diabetic animals. By contrast, some studies indicate that Win 55,212-2 antinociception remained intact in diabetic animals (Ulugol et al 2004; Hama and Sagan, 2007; Comelli et al., 2009). This discrepancy may be possibly due to the use of a different diabetic model and duration of study.

A significant reduction in DAMGO and morphine (mu-opioid–agonist) induced antinociceptive effect was also observed, which may be due to decrease in mu-opioid receptors in spinal cord, in diabetic mice. In contrast to this, the analgesics effect of DPDPE (k-agonist) and oxycodone was not affected in diabetic animals. This differential modulation of analgesics effect of opioids may be due to impaired activation or desensitization or down regulation of mu-opioid receptor, while the k-receptor remained unaltered in diabetic animals. Similarly, development of tolerance to NSAIDs, cannabinoids and neurosteroids was observed in diabetic mice, as compared to non-diabetic mice.

Hyperglycemia modulates several enzymes, neurotransmitters and endogenous mediators involved in regulation of pain sensitivity, including PKC and NMDA (Danis and Sheetz, 2009; Chen et al., 2009). Hyperglycemia induced activation of PKC is known to contribute in painful diabetic neuropathy (PDN) (Evcimen and King, 2007). PKC phosphorylates numerous receptors and ion channels, including the mu-opioid and CB-1 cannabinoid receptor and the calcium channel associated with the NMDA receptor. PKC-mediated phosphorylation of the NMDA receptor, that expels the Mg$^{2+}$ ion that at rest blocks the Ca$^{2+}$ channel (Chen and Huang, 1992). The unblocked channel no longer requires depolarization for activation, resulting in a
positive feedback loop of amplified NMDA-receptor responses and further activation of PKC. Phosphorylation of the mu-opioid and CB-1 cannabinoid receptor by PKC may uncouple the receptor from its G-protein or alter the properties of its associated potassium channel. In either case, the result is reduced responsiveness of the mu-opioid and cannabinoids-CB-1 receptor to exogenous opioid and cannabinoids agonist.

Further, a recent study has shown that hyperglycemia and activation of PKC decreases mu-opioid and cannabinoids CB-1-receptor mRNA levels in spinal cord and dorsal root ganglion, suggesting that PKC also inhibits mu-opioid and cannabinoids CB-1 receptor turnover in diabetic patients (Chen et al., 2002; Zhang et al., 2007). In addition, PKC is known to be involved in mu and CB-1 receptor desensitization and to potentiate analgesics tolerance (Bailey et al., 2009; Hull et al., 2010). Therefore, it is possible that activation of PKC by persistent hyperglycemia is involved in development and maintenance of hyperalgesia and allodynia and analgesics tolerance observed in STZ-treated animals. This has been confirmed in the present study, PKC inhibitor chelerythrine and low dose NAL and NTX opioids and cannabinoids receptor antagonist RIM were found to reverse STZ-induced opioids and cannabinoids tolerance. This suggests that direct PKC activation by hyperglycaemia mediated mu-opioid and CB-1 cannabinoid agonists, is involved in the processes that result in mu and CB1-receptor down-regulation in PDN and that conventional PKC isozymes are involved in maintenance of PDN. The involvement of PKC in the development of opioids tolerance is well established (Ohsawa and Kamei, 1997; Hull et al., 2010). Thus the present study for the first time indicates the involvement of PKC in cannabis and Win 55, 212-2 induces tolerance in diabetic mice, which may be
due to either PKC mediated phosphorylation of CB1-receptor or rapid CB1-receptor desensitization.

The mechanism of PDN is multifaceted and not well established. However, oxidative stress and cytokines are well studied mechanisms involved in PDN (Ozkul et al., 2010). In this study, lipid per-oxidation, a marker of oxidative stress was significantly increased following STZ-injection. Also, the levels of anti-oxidant enzymes, including SOD, GSH and CAT were significantly reduced in STZ treated rats and mice.

Spinal pro-inflammatory cytokines are powerful pain-enhancing signals that contribute to neuropathic pain (Malin et al., 1996; Doupis et al., 2009). Following nerve injury, activation of immune-like glia cells such as astrocytes and microglia has been reported, and may contribute to hyperalgesia, mechanical allodynia or chronic inflammatory pain in animal models (Tsuda et al., 2005; Ledeboer et al., 2007). Microglia cells activation following nerve injury, releases diffusible substances from neurons such as NO, fractalkine, substance P, and excitatory amino acids that consequently increase the release of cytokines and PGs (Inoue et al., 2007). These inflammatory agents, mainly cytokines, have been shown to activate and/or enhance the sensitivity of primary afferents and spinal cord neurons. Therefore, STZ-induced hyperalgesia and allodynia was thought to result exclusively from altered neuronal activity in the primary sensory and spinal cord neurons, probably via the release of neuro-active diffusible factors including prostanoids and cytokines (Doupis et al., 2009). Further, oxidative stress and pro-inflammatory cytokines (TNF-α, IFN-γ and IL-1β) are well documented to modulate nociceptive threshold and are thought to be involved in ; a) STZ-induced decreased sensitivity to antinociceptive effect of morphine (Yu et al., 2007; Taliyan et al., 2010a & b) and cannabinoids receptors
(Zhang et al., 2007) and b); up-regulation of COX-expression, including COX-2, and modulation of their actions (Ragvendra et al., 2002).

Other markers of inflammation are activation of NF-kB and NF-kB derived pro-inflammatory cytokines, including TNF-α, that are elevated by diabetes in the peripheral nerves (Guha et al., 2000; González-Clemente et al., 2005) and that either COX-gene inactivation or pharmacological COX blockade prevents this increase (Kellog et al., 2007b). Moreover, NF-kB can be further activated by increased mitochondrial superoxide, non-mitochondrial ROS production (Newsholme et al., 2007), TNF-α up-regulation (Jobin et al., 1998; Moller, 2000), and RAGE signalling in diabetes (Sousa et al., 2000). Therefore, in diabetes, COX–derived PGE2, in concert with increased oxidative/nitrosative stress and RAGE activation may create a positive feedback loop that leads to increased oxido-nitrosative stress and inflammation in the diabetic peripheral nerves and possibly COX-induced vicious cycle is involved in pain hypersensitivity (Shanmugam et al., 2006). A significant decrease in the effectiveness of COX inhibitors in PDN reported earlier (Courteix et al., 1993), was also observed in the present study. It is possible that oxido-nitrosative stress induced by COX activation may be responsible for the decrease in analgesic effect.

In the present study, by the end of 3rd week, diabetic rats exhibited hyperalgesia and allodynia and showed a significant increase in nitrite concentration and exhibited analgesic tolerance to various analgesics, as compared with control animals. This indicates that uncontrolled glial cell activation after nerve injury and the increase released of cytokines, is a key player in hypersensitivity of neuron in diabetic patients. Moreover, hyperglycemia stimulates the production of AGEs, activates PKC, and enhances the polyol pathway leading to increased superoxide anion formation, which
plays an important role in the development of PDN (Friederich et al., 2009; Ozkul et al., 2010)

Hyperglycaemia-induced superoxide generation contributes to the increased expression of NAD(P)H oxidase, which in turn generate more superoxide anion (Wautier et al., 2001; Ushio-Fukai, 2006). Hyperglycaemia also favours’, through the activation of NF-κB an increased expression of iNOS, which may increase the generation of NO (Xie et al., 1994; Flodstrom et al., 1996). Moreover, pro-inflammatory cytokines are known to induce the expression of iNOS (Deng et al., 1993). Superoxide anion interacts with nitric oxide, forming the powerful oxidant peroxynitrite (ONOO−), and the generated cytotoxin peroxynitrite, which attacks various biomolecules in the vascular endothelium, vascular smooth muscle and myocardium, neuronal cells, leading to PDN via multiple mechanisms (Salvemini et al., 2009).

Increased nitrosative stress has been documented in vascular endothelium (Pacher et al., 2005; Szabo et al., 2002b), myocardium (Pacher et al., 2005), retina (Du et al., 2002), and kidneys (Drel et al., 2006a & b) of STZ-diabetic animals and human subjects with diabetes (Afanasev et al., 2010). Increased nitrotyrosine immunoreactivity has also been demonstrated in peripheral nervous system i.e., peripheral nerve, spinal cord and dorsal root ganglion (DRG) neurons, of STZ-diabetic rats (Cheng and Zochodne, 2003; Obrosova et al., 2005) and STZ-diabetic (Ho et al., 2006; Obrosova et al., 2005a & 2005b), ob/ob (Drel et al., 2006), and high-fat diet fed mice (Obrosova et al., 2007), and epineurial vessels of STZ-diabetic, ZDF diabetic fatty, and Zucker fatty rats (Obrosova et al., 2005b; Oltman et al., 2005). Furthermore, increased nitrosylated protein has been identified very early during exposure of cultured human Schwann cells to high glucose (Radi, 2004). Moreover,
increased nitrotyrosine immunoreactivity in microvasculature of type 2 diabetic patients (Thuraiasingham et al., 2007) was shown and a good correlation was observed between nitrotyrosine immunostaining intensity and fasting blood glucose, HbA1c, intracellular adhesion molecule (ICAM), and vascular cellular adhesion molecule (VCAM).

Some studies implicate peroxynitrite in motor and sensory nerve conduction deficits, thermal hypoalgesia, and impaired nitrergic innervation, in STZ-diabetic rats and mice. These neuropathic pain behaviours symptoms of STZ-diabetic rats and mice were normalized by peroxynitrite decomposition catalyst agents such as FeTMPS [5,10,15,20-tetrakis(2,4,6-trimethyl-3,3-disulfonatophenyl)-porphyrinato iron (III)], FP-15 or WW85 (Szabo et al., 2002; Nomiyama et al., 2004; Duplain et al., 2008). In addition, peroxynitrite has been implicated in the development of antinociceptive tolerance and co-administration of sub-effective dose of FP-15 with morphine, markedly enhanced antinociception (Muscoli et al., 2007). Thus, it seems that hyperglycemia enhanced NO or peroxynitrite formation, via multiple mechanisms may be involved in development of tolerance to various analgesics. In the present study, administration of minocycline, (glial cells inhibitor), cyclosporine-A, (an interleukin-2 inhibitor), thalidomide (TNF-α inhibitor), and pentoxifylline (a cytokines inhibitor), significantly prevented diabetes-induced thermal hyperalgesia and mechanical allodynia. Moreover, treatment with these drugs attenuated the increase in nitrite levels. Further, L-arginine, a nitric oxide donor, abolished the protective effect of these drugs. These results indicates that overproduction of ROS and cytokines by persistent hyperglycemia-induced NO generation was, a key player involved in the development of hyperalgesia and allodynia in diabetic animals.
The non-obese diabetic mouse provides a relevant model for insulin-dependent diabetes mellitus (IDDM). The role of autoimmune mediated destruction of islet of β-cells is indicated by islet infiltration by mononuclear cells, mainly T-lymphocytes and macrophages (Itoh et al., 1993; Sami et al., 1999). These mononuclear cells may cause this destruction either directly or through the secretion of proinflammatory cytokines, such as interleukin-1β and interferon-γ, and free radicals (Bach, 1995; Sandler et al., 2000). It has been shown that prevention of IDDM is possible with neonatal thymectomy or splenectomy (Boitard et al., 1991), and by in vivo/vitro treatment with cytokines inhibitor such as cyclosporine /thalidomide or cytokines antibodies, anti-CD4 +, or anti-I-A m Abs (Kamaie et al., 1994; Taliyan et al., 2010). Moreover, treatment of overtly diabetic NOD mice with anti-lymphocyte serum (ALS), a polyclonal anti–T-cell antibody, abrogates autoimmunity and achieves partial clinical remission of DM (Chatenoud et al., 1997).

Thymus gland and spleen are the principle organs of immune system and a rich source of cytokines. Thymectomy has been reported to provide beneficial effect in advanced Type-1 Diabetes Mellitus (IDDM) but is associated with potential toxicity (Like et al., 1982; Jeroen et al., 2004). Spleen is a rich source of cytokines, and the spleen derived factor has been reported to modulate antinociceptive effect of morphine (Kamei et al., 1998). Therefore, it is possible that the observed decrease in antinociceptive effect of various analgesics in diabetic rats may due to an increased formation and release of factor(s) from mononuclear cells of spleen.

In the present study, administration of SHS of non-diabetic mice and heated fraction of SHS of diabetic mice for 28 days in non diabetic mice did not affect pain latency. However, non-heated fraction of SHS of diabetic mice (28 day), injected in non diabetic animals, produced hyperalgesia and allodynia similar to that observed in
diabetic animals. This indicates that the factor(s) synthesized and released from spleen mononuclear cells were peptide in nature and may be cytokines or cytokines-like substances. Injection of SHS of diabetic mice, significantly increased the nitrite level in recipient non-diabetic mice, indicating the involvement of spleen or spleen mononuclear cells of diabetic rats in decreasing analgesic effect of various analgesics that seems to be mediated through increased expression of iNOS. Splenectomy significantly attenuated the diabetes-induced increase in nitric oxide level in both urine and serum test. The involvement of cytokines was further confirmed by using cyclosporine-A, thalidomide, pentoxifylline and minocycline treatments in diabetic and the drug treated SHS (28 day), prevented hyperglycemia–induced hyperalgesia and allodynia and also markedly attenuated NO level. Administration of these drugs also potentiated analgesics effect of various analgesics. It seems that preventive effect of these drugs was due to the decrease in cytokines or cytokines-like inflammatory mediators and the resultant increase NO production. Thus, the present study for the first time, has provided the evidence of spleen or spleen derived factor (S) involvement in diabetes-induced decrease in analgesic efficacy of Win 55,212-2, NSAIDs and neuroseriods. It is suggested that splenectomy may be a useful approach to manage difficult to treat neuropathy.

Aminoguanidine (AG) and L-NAME, iNOS inhibitors, significantly inhibited and reversed the established hyperalgesia and allodynia in diabetic animals. This confirmed that STZ-induced hyperalgesia and alldynia was the result of overproduction of NO However, other mechanism such as inhibition of AGE formation by AG was not excluded.

AGE products have been reported to activate transcription factor nuclear factor \( \kappa B \) (NF-\( \kappa B \)) that regulates the gene expression of growth factors and cytokines (Cameron
et al., 2008; Maryam and Zeinab, 2010). Moreover, AGEs have been demonstrated to enhance the expression of inducible nitric oxide synthase (iNOS) by stimulating the expression of pro-inflammatory cytokines such as TNF-alpha in diabetic rats and also to stimulate NMDA receptor (Sugimoto et al., 1999). Activation of NMDA receptor enhanced neuronal hypersensitivity and has been implicated in PDN (Chen et al., 2010). Co-administration of ketamine and dextromethorphan for two weeks, with analgesics in the present study, significantly reversed STZ-induced decrease in the antinociceptive effect of various analgesics and also attenuated the increase in nitrite levels, indicating that activation of NMDA by persistent hyperglycemia increase the production of nitric oxide.

Thus convincing evidence is available from several published studies, that hyperglycemia-induced multiple mechanisms, converge on induction of iNOS and the formation of NO and or –ONOO, which may be involved in hyperglycemia induced tolerance to analgesic action of morphine. The results obtained in the present study, not only lend further support to this hypothesis, but also extend it to the hyperglycemia-induced tolerance to the analgesic action of NSAIDs, cannabinoids and neurosteroids. Therefore inhibitors of iNOS, peroxynitrite formation, and catalytically based increased peroxynitrite degradation are potential targets, for new drug discovery, to manage PDN.

**Lacuna in the present study**

NO level was measured by indirect method (nitrate/nitrite) levels in serum and tissues, due to lack of facility for direct measurement of NO level.
Suggestion for future work in this area

To characterize the nature and quantify the substance(s) present in spleen and SHS of diabetic rats