Kafkas Univ Vet Fak Derg
19 (4): 583-587, 2013
DOI: 10.9775/kvfd.2012.8416

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

The Effects of D-Ala^2, D-Leu^5-Enkephalin (DADLE)'s Continuous Perfusion on Erythrogram and Biochemical Profile in Rats [1]

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[1] *This study is has been supported by the Research Fund of Istanbul University (Project No: 102/15052003)*

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Makale Kodu (Article Code): KVFD-2012-8416

Summary

In this experimental study it was aimed to investigate the effects of chronic continuous DADLE administration on erythrogram and the biochemical profile (ALP, ALT, AST, LDH, glucose, urea, creatinine, albumin, total protein). The study was carried on 48 adult male Wistar rats. DADLE was administered via osmotic mini-pumps, implanted subcutaneously. After the 28-day treatment, plasma glucose levels increased and urea levels decreased significantly in experimental group animals compared to the control group. Differences between the groups were not found to be statistically significant with respect to the other investigated parameters. So we conclude that chronic continuous administration of DADLE via osmotic mini-pumps did not exert any detrimental effect on erythrogram and the biochemical profile in rats.

Keywords: *DADLE, Opioid, Osmotic pump, Erythrogram, Biochemical profile*

D-Ala^2, D-Leu^5-Enkephalin (DADLE)'in Sıçanlarda Sürekli Perfüzyonunun Eritrogram ve Biyokimyasal Profile Etkisi

Özet

Bu deneysel çalışmada DADLE'nin kronik sürekli uygulamasının bazı eritrositer parametreler (eritrogram) ve biyokimyasal profil (ALP, ALT, AST, LDH, glikoz, üre, kreatinin, albümin, total protein) üzerine olan etkilerinin araştırılması amaçlanmıştır. Çalışmada 48 adet yetişkin erkek Wistar sıçan kullanılmıştır. DADLE deri altına implante edilen ozmotik mini pompalar ile uygulanmıştır. Yirmisekiz günlük uygulama sonrası deney grubu hayvanlarının plazma glikoz düzeyleri artarken üre düzeyleri düşmüş ve bu değişikliklerin kontrol grubu ile karşılaştırıldığında istatistiksel önemde olduğu görülmüştür. İncelenen diğer parametreler açısından gruplar arasındaki farklar istatistiksel olarak önemli bulunmamıştır. Bu nedenle DADLE'nin ozmotik mini pompalar ile kronik ve sürekli uygulanmasının sıçanlarda eritrogram ve biyokimyasal profil açısından herhangi bir zararlı etki oluşturmadığı sonucuna varılmıştır.

Anahtar sözcükler: *DADLE, Opioid, Ozmotik pompa, Eritrogram, Biyokimyasal profil*

INTRODUCTION

Hibernation is an adaptive and protective strategy for a group of animal species and makes it possible for them to survive extended periods of food deprivation and extreme ambient conditions [1,2]. Animals in the hibernating state can tolerate body temperatures close to zero [3] and excessively decreased blood flow to vital organs [4,5].

Dawe and Spurrier [6] showed that blood from hibernating animals can induce hibernation when transfused into active euthermic individuals and suggested for the first time the existence of a triggering substance in the blood. Furthermore they showed that this hibernation induction trigger (HIT) is not speciesspecific [7]. Later it has been found that HIT is present in the albumin fraction of plasma [8] and opioid receptors are involved in the induction of hibernation [9]. The synthetic peptide DADLE (D-Ala^2,D-Leu^5-Enkephalin; $C_{29}H_{39}N_5O_7$) is a stable analogue of the endogenous delta opioid encephalin [10,11]. It passes blood-brain barrier after systemic application [12] and has a selective agonistic

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property on delta-opioid receptors [10]. DADLE has been found to induce hibernation in hibernant animals after continuous infusion [13].

The fascinating tissue protective adaptation seen during hibernation attracted substantial research interest on HIT and DADLE, so a wide range of studies have been conducted investigating the potential protective efficacy of them [4,14]. Indeed it has been demonstrated that HIT and DADLE prolong the survival of isolated organs (heart and liver) in a multiorgan preservation preparation almost 6-fold, which could have crucial implications for organ transplantation research [15,16]. Summers et al.[17] found that DADLE treatment improves hemodynamic stability and prolongs survival in an animal model of severe hemorrhagic shock. Another *in vivo* study showed that parenteral administration of DADLE protects the liver of rats against ischemia reperfusion injury [18]. Further studies pointed out the protective efficacy of DADLE on the nervous system [10,11,19]. Recently, Borlongan et al.[20] showed that DADLE pre-treatment, administered parenterally, prevents cell death processes and behavioral symptoms caused by experimentally induced stroke in rats.

The aforementioned results together manifest that DADLE has substantial protective efficacy in various cells and tissues and may be regarded as a promising novel therapeutic agent. Nevertheless it is known that prevalence of anemia is highly correlated with opioid use [21], and it has been reported that opioids cause membrane alterations of red blood cells [22]. Besides activation of different opioid receptor subtypes may exert different effects on target cells [23]. To the best of our knowledge no study has so far investigated the side effects of DADLE on erythrogram or the biochemical profile. So the aim of the present study was to investigate the potential side effects of long-term DADLE infusion on some erythrocyte and biochemical parameters.

MATERIAL and METHODS

Animals

Male Wistar albino rats weighing 250-275 g were used for the study. They were randomly allocated to 2 groups, each consisting of 24 rats, as control and experimental groups. All animals were housed in polycarbonate cages with wood shavings bedding in a climate-controlled animal room with 12/12 h light/dark cycle. They were fed standard laboratory chow and tap water ad libitum. All experimental procedures were approved by Istanbul University Veterinary Faculty's Animal Experiments Local Ethics Committee (Approval no. 2003/65)

DADLE Application

Rats were anesthetized using xylazine/ketamine (10/75 mg/kg). Osmotic mini pumps (Alzet, USA) containing DADLE (Sigma-Aldrich, Germany) and physiologic saline solution were implanted subcutaneously into the interscapular region of experimental and control group animals, respectively. Experimental group animals received approximately 2.5 µl (±10%) of DADLE solution (0.5%) per hour, depending on the mean pumping rate of osmotic pumps during 28-day experimental period.

Samples and Tests

At the end of the experimental period all animals were exsanguinated via cardiac puncture under general anesthesia with xylazine/ketamine (10/75 mg/kg, Bayer, Turkey). Blood samples were collected into dry and EDTA tubes. Erythrogram parameters were determined using anticoagulated blood samples by using an electronic cell counter (Medonic 570, Boule Medical, Sweden) . Sera were obtained from blood samples collected into dry tubes and were used for the determination of biochemical profile including following parameters: alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose (GLU), creatinine (CREA), lactate dehydrogenase (LDH), albumin (ALB), total protein (TP) and urea (URE) using an automatic biochemical analyzer (Abbott Architect, Abbott Laboratories, USA).

Statistical Analysis

All statistical analyses were performed with statistical software SPSS (version 11.5.2.1) using the independent samples t-test. All results are reported as means ± SEM. Significance was accepted at $P<0.05$ level.

RESULTS

Hematological parameters are shown in *Table 1*. There were no statistically significant differences between groups.

The data on biochemical parameters are shown in *Table 2*. Glucose level increased and urea level decreased in DADLE treated group compared to the control group, and both differences were found to be statistically significant ($P<0.05$). There were no statistically significant differences regarding other parameters.

Table 1. *Hematological parameters*
Tablo 1. *Hematolojik parametreler*

Parameter	Control (n=24)	DADLE (n=24)
Red blood cells (10^6/mm^3)	7.91±0.11	8.08±0.09
Hemoglobin (g/dl)	12.27±0.16	12.44±0.12
Hematocrit (%)	40.98±0.54	41.49±0.40
MCH (pg)	15.52±0.15	15.43±0.12
MCHC (g/dl)	29.96±0.11	30.03±0.08
MCV (fl)	51.81±0.42	51.40±0.41
RDW (%)	21.62±0.31	21.29±0.32

MCH: *mean corpuscular hemoglobin;* ***MCHC:*** *mean corpuscular hemoglobin concentration;* ***MCV:*** *mean corpuscular volume;* ***RDW:*** *red blood cell distribution width*

Table 2. *Biochemical parameters*
Tablo 2. *Biyokimyasal parametreler*

Parameter	Control (n=24)	DADLE (n=24)
ALP (U/L)	387.61±14.63	409.13±22.40
ALT (U/L)	79.17±3.31	84.75±3.01
AST (U/L)	154.17±12.07	164.83±16.59
LDH (U/L)	362.86±9.96	392.29±16.65
Urea (mg/dl)	37.57±0.74*	34.54±0.88*
Albumin (g/dl)	3.19±0.05	3.17±0.03
Glucose (mg/dl)	161.65±4.07*	178.71±4.42*
Creatinine (mg/dl)	0.40±0.01	0.40±0.01
Total protein (g/dl)	6.92±0.06	6.95±0.07

ALP: *alkaline phosphatase;* ***ALT:*** *alanine aminotransferase;* ***AST:*** *aspartate aminotransferase;* ***LDH:*** *lactate dehydrogenase; * Differences between the means of control and experimental groups are statistically significant (P<0.05)*

DISCUSSION

To the best of our knowledge there are no studies examining the effects of long-term DADLE administration on erythrogram. In the present study 28-day continuous infusion of DADLE did not reveal any significant effect on the investigated parameters related to erythrocytes. Although studies focusing on the side effects of DADLE on hematological parameters are lacking, the effects of other opioids such as morphine on erythrocytes and related parameters have been investigated in various studies. Nie et al.[24] found that acute and chronic *in vivo* and *in vitro* application of morphine, a mu-opioid receptor agonist, increased the osmotic fragility of red blood cells and shortened their lifetime by affecting the cell membrane. Zeiger et al.[22] explained the high anemia prevalence among chronic opioid users with increasing levels of opioid receptors on red blood cells due to chronic opioid usage and subsequently increasing deformability of erythrocytes. Even though there is evidence indicating the presence of kappa- and mu-opioid receptors on the erythrocyte membrane [22,25], we could not encounter any finding about the delta opioid receptors in the literature. The abovementioned detrimental effects of opioids on erythrocytes were attributed both to the ligand-receptor interactions [22] and to the direct effect of opioid on erythrocyte membrane [24]. So the absence of any effect of DADLE on red blood cells and related parameters can be due to the lack of delta opioid receptors on erythrocyte membran or could be interpreted that chronic DADLE administration at this dose does not have any side effects on erythrocyte membran directly.

Because of its crucial role in biotransformation and metabolism of most opioids as well as various other drugs, liver is predisposed to toxic injury, especially in chronic exposure to these drugs [26-28]. Indeed it has been reported that parenteral administration of various opioids caused hepatic damage indicated by increased liver enzymes [29]. However to the best of our knowledge there are no studies investigating the adverse effects of chronic DADLE treatment on liver. Notwithstanding with the reports about detrimental effects of opioids on liver, Yamanouchi et al.[18] stated that acute DADLE treatment exerted a hepato-protective effect against acute liver damage and decreased serum ALT level in rats. In the present study chronic DADLE treatment via osmotic mini-pumps showed neither a beneficial nor a detrimental effect on liver enzymes.

Lactate dehydrogenase is a stable cytosolic enzyme present in a wide variety of mammalian tissues and cells, and leaks out of the cells into the bloodstream as a result of cell membrane damage [30]. The increase of LDH level in extracellular fluid (e.g., plasma, serum) is a sensitive but nonspecific marker of cell and tissue damage including liver, kidney, heart and muscle [31,32]. There are various *in vitro* studies in literature, investigating the effects of DADLE on several tissues and cells and it has been reported that DADLE exerted protective effect on these tissues and cells indicated by a decrease of LDH level [33-36]. Dalargin (D-Ala^2,Leu^5,Arg^6-enkephalin), another delta opioid receptor (DOR) agonist similar to DADLE, has been found to have a decreasing effect on LDH level in a traumatic shock model in rats [37]. Atıcı et al.[28] reported that long-term opioid treatment caused hepatic and renal damage in rats as indicated by a serum LDH increase. Nevertheless we could not find any reports about the *in vivo* effects of chronic DADLE treatment on LDH level. In the present study long-term DADLE treatment did not alter the plasma LDH activity, and this finding can be interpreted as an absence of detrimental effects on tissues including liver and kidney.

There was no significant difference between the creatinine levels of experimental and control groups in the present study. Besides, plasma urea level of DADLE treated animals was significantly ($P<0.05$) lower than that of the control group animals. Nevertheless both parameters were in normal range. Since the DADLE treatment did not alter the creatinine levels of both groups, it can be argued that the glomerular function was not altered by long-term treatment. So the aforementioned decrease in urea level of the experimental group can be based either on the decrease of protein catabolism or on an impairment of urea synthesis. The first possibility of altered protein metabolism by DADLE can be eliminated considering that there was no difference between plasma albumin and total protein levels of groups, and both parameters were in normal range. In this context it can be suggested that the decrease in the urea level of the experimental group is based on its impaired synthesis in liver. However we could not find any data about the effects of long-term DADLE treatment on urea synthesis in the literature to compare and discuss this possibility.

A wide range of studies has been conducted to investigate the effects of opioids on glucose homeostasis [38-44].

It has been known for several decades that parenterally administered morphine causes hyperglycemia [45]. Bailey and Flatt [42] reported that mu receptor agonist DAMGO (D-Ala², N-Me-Phe⁴, Gly⁵-ol-Enkephalin) and delta receptor agonist DADLE dose dependently increased plasma glucose levels in rats. Beta endorphin, which exhibits high affinity for delta opioid receptors [46], has been found to significantly increase plasma glucose level [47]. Nonetheless Gunion et al.[39] reported that only mu-opioid receptor stimulation resulted in hyperglycemia in rats whereas delta opioid receptor sub-type was ineffective. In our present study long-term DADLE treatment produced a significant (P<0.05) hyperglycemia in the experimental group. This increasing effect of DADLE on plasma glucose level may be due to its interaction with delta opioid receptors as suggested before [40-42].

In summary 28-day continuous infusion of DADLE via osmotic mini-pumps in our present study did not cause any significant alterations on erythrocyte parameters and biochemical profile except for a decrease in the plasma urea level and an increase in the glucose level. The finding of hyperglycemia is in accordance with previous data. Even though there is no relevant data in the literature to compare and discuss the significant decrease in the plasma urea level that we have observed, the levels of experimental and control groups were in normal range. Considering that the inspected biochemical parameters related to liver and kidney functions and the erythrocyte parameters were unaffected by DADLE, it would be reasonable to suggest that DADLE does not have any major detrimental effects at this dose level. Because of its promising tissue protective effects, DADLE deserves further study as a therapeutic agent, and our results could contribute to the further evaluation of chronic effects of DADLE treatment on various tissues, organs and systems.

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