TÜBİTAK

Research Article

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Chronic continuous perfusion of the hibernation triggering opioid DADLE did not cause any major physiological alterations in rats

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Abstract: [D-Ala^2, D-Leu^5] enkephalin (DADLE), a delta opioid receptor agonist, can induce mammalian hibernation and has hibernation-like effects in non-hibernating species. It has also been demonstrated that DADLE has protective efficacy on various tissues such as nervous, cardiac, and hepatic tissues. Despite substantial research conducted in the last decade about the tissue protective efficacy of DADLE, detailed underlying mechanisms have not been fully elucidated. In the present study we investigated in a rat model whether continuous perfusion of DADLE can induce potentially tissue protective alterations in physiological parameters. In the study 48 young male Wistar rats were used. They were divided into control and experimental groups, each group consisting of 24 animals. DADLE was perfused to experimental group rats continuously for 28 days using subcutaneously implanted osmotic mini-pumps. We investigated its effects on body temperature, antioxidant protection [total antioxidant capacity (TAC), ascorbic acid (AA), and malondialdehyde (MDA) levels], white blood cell (WBC) count, and coagulation parameters [activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT)]. While thrombin time was significantly lengthened in the DADLE treated group, there were no statistically significant differences in the other investigated parameters between the experimental group and the control group.

Key words: DADLE, opioid, hibernation, osmotic mini-pumps, rats

Hibernasyonu tetikleyici opioid DADLE'nin kronik sürekli perfüzyonu sıçanda majör bir fizyolojik değişikliğe neden olmadı

Özet: [D-Ala^2, D-Leu^5] enkephalin (DADLE), memeli hibernasyonunu başlatabilen ve hibernant olmayan türlerde de hibernasyon benzeri etkilere neden olabilen bir delta opioid reseptör agonistidir. Ayrıca DADLE'nin sinir, kalp ve karaciğer dokusu gibi çeşitli dokular üzerinde koruyucu etkinliğinin olduğu gösterilmiştir. DADLE'nin doku koruyucu etkinliği üzerine son yıllarda yürütülen çok sayıda araştırmaya rağmen, bu etkinliğin altında yatan detaylı mekanizma tam anlamıyla aydınlatılamamıştır. Biz bu çalışmada DADLE'nin sürekli perfüzyonunun sıçanda fizyolojik parametrelerde potansiyel olarak doku koruyucu etkiler oluşturup oluşturamayacağını araştırdık. Çalışmada 48 adet erkek Wistar sıçan kontrol ve deney grubu olarak 24'erli iki gruba ayrıldı. DADLE, deney grubu hayvanlarına deri altına implante edilmiş ozmotik mini pompalar ile 28 gün boyunca sürekli perfüze edildi. DADLE'nin vücut ısısı, antioksidan savunma sistemi (total antioksidan kapasitesi (TAC), askorbik asit (AA), malondialdehit (MDA) düzeyleri), lökosit sayısı (WBC) ve koagulasyon parametreleri [active parsiyel tomboplastin zamanı (APTT), protrombin zamanı (PT), trombin zamanı (TT)] üzerine etkisi incelendi. Deneme grubunda trombin süresi anlamlı olarak uzarken, incelenen diğer parametreler açısından her iki grup arasında istatistiksel düzeyde anlamlı fark bulunmadı.

Anahtar sözcükler: DADLE, opioid, hibernasyon, ozmotik mini-pompa, sıçan

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Introduction

Hibernation is a physiological phenomenon that consists of various physiological alterations such as hypothermia, long-term aphagia, bradycardia, and metabolic depression, which certain animal species experience when they are exposed to the extremes of cold and limited food supplies (1,2). Hibernation represents a unique physiological adaptation that allows animals to survive in challenging natural conditions such as extended periods of food deprivation and extreme cold weather (3).

Physiological parameters of animals show extreme variations during the hibernation period; colonic temperatures fall to as low as –1.3 °C (4), basal metabolic rate decreases to 1%, oxygen consumption decreases to 50%, respiration rate decreases to 1-2 respiration per minute, and heart rate decreases to 3-10 beats per minute (5). Non-hibernating species cannot tolerate these extreme variations in physiological parameters. Hibernation is a natural adaptation mechanism useful in conditions that can cause neural loss and brain damage in non-hibernating animals within minutes, especially considering significantly reduced blood circulation and oxygen level in brain tissue (3,6).

There is a "triggering substance" or hibernation induction trigger (HIT) in the blood of hibernating mammals, which is responsible for natural hibernation and can induce hibernation in euthermic individuals of the same species via blood transfusion (7). It was found that HIT is not species specific (8) and has a wide range of physiological effects on non-hibernating species (1,9). Oeltgen et al. (2) found that DADLE, which is a delta opioid receptor agonist, induces hibernation in summer-active ground squirrels in a manner comparable to that induced by HIT. Similar to HIT, direct infusion of DADLE leads to hibernation-like effects such as respiratory depression, analgesia, hypotension, and decreased heart rate in non-hibernators (9).

Both HIT and DADLE have been reported to prolong tissue survival time of various organs such as the heart, lung, liver, and kidney (10,11). Yamanouchi et al. (12) reported that DADLE protects the liver against ischemia/reperfusion (IR) injury in rats. Moreover, it has been shown that DADLE has neuroprotective efficacy in the central nervous system, and it has been suggested that DADLE might be of important clinical value in the therapies of neurodegenerative diseases (13-15). Despite substantial research conducted in the last decade about the tissue protective efficacy of DADLE, detailed underlying mechanisms have not been fully elucidated.

Previous research investigated DADLE's general tissue protective effects in vivo and in vitro with both acute and chronic applications (12-16). Most of the research mentioned above focused on the protective efficacy of DADLE against potentially damaging harmful impacts from various sources such as toxic agents, asphyxia, ischemia, and hemorrhagic shock. On the other hand, the effects of DADLE on various physiological parameters without exposure to any harmful impact are worth investigating to elucidate the underlying mechanisms of DADLE's protective efficacy. To the best of our knowledge, no study has investigated the effects of continuous DADLE infusion on physiological parameters of a non-hibernating species to date. In this context, in the present study we aimed to investigate the effects of long-term continuous infusion of DADLE (rate of 2.5 μL/h, 0.5% DADLE solution) in rats. Tissue protective efficacy of DADLE has been mainly attributed to decreasing lipid peroxidation and to preventing free radical formation (13,16). Therefore, the core of investigated parameters in this study has consisted of the parameters related to the antioxidant defense system: malondialdehyde (MDA) levels as an indicator of lipid peroxidation, total antioxidant capacity (TAC), and plasma ascorbic acid level (AA). Additionally, since DADLE leads to hibernation-like effects in non-hibernators (9), physiological and hematological parameters related to the potentially neuroprotective aspects of hibernation were also monitored. These are hypothermia (body temperature), immunomodulation (WBC count), and hypocoagulation (APTT, PT, TT).

Materials and methods

Forty-eight young male Wistar rats (mean body weights: 250.09 ± 24.59 g) were used in the study. They were housed individually with water and commercial rat pellets available ad libitum and were maintained on a 12/12 h light/dark cycle. The rats were

divided into 2 groups as control and experimental groups, and each group consisted of 24 animals. Body temperatures of animals were measured and recorded daily during the experimental period. All experimental procedures were approved by İstanbul University Veterinary Faculty's Ethics Committee.

Mini osmotic pumps (2ML4, Alzet-Durect, Cupertino, USA) containing 0.5% DADLE solution and physiologic saline were implanted subcutaneously into the interscapular region of experimental and control animals, respectively, under xylazine/ketamine (10/75 mg/kg) anesthesia at the first day of the experimental study. Experimental group animals received 2.5 μL, 0.5% DADLE solution subcutaneously per hour (the mean pumping rate of osmotic pumps) during the 28-day experimental period.

At the end of the experimental period, blood samples were collected via cardiac puncture under xylazine/ketamine (10/75 mg/kg) anesthesia, and the animals were sacrificed. Coagulation screening tests (APTT, PT, and TT) and leukocyte counts were performed in citrated and heparinized blood samples, respectively. APTT, PT, and TT were measured using commercial kits (Diagnostica Stago, France) by magnetic coagulometer (Amelung, KC1, Germany). Leukocyte counts were determined by an automated cell counter (Medonic 570, Sweden).

The remaining amount of heparinized blood was centrifuged, and plasma was separated to be used for determination of TAC, AA, and MDA levels.

Brain tissues of animals were removed, washed in ice-cold buffer (140 mM KCl, 20 mM Tris- HCl, pH 7.4) and homogenized with the same buffer solution. Homogenates were centrifuged (20,000 ×*g* / 4 °C /10 min), and TAC, total protein (TP), and MDA levels were measured in supernatants.

MDA level was measured by monitoring thiobarbituric acid reactive substance formation according to the method described by Yoshioka et al. (17). TAC levels of brain tissue and plasma were measured by an automated analyzer (Aeroset, Abbott, USA) using kits developed by Erel (18).

Total protein concentration of brain tissue was estimated by using the method of Lowry et al. (19). TAC and MDA levels of brain tissue are expressed as nmol/mg and mmol trolox equiv./mg of tissue protein, respectively.

The plasma level of ascorbic acid was detected spectrophotometrically using the method of Haag (20).

All statistical analyses were performed with SPSS (version 10.0) using the independent samples t-test. Results are reported as means ± SEM for the comparison between study and control group values. Significance was accepted at $P < 0.05$ level.

Results

The daily mean body temperatures of the groups are presented in Figure 1. There was no significant difference between the groups.

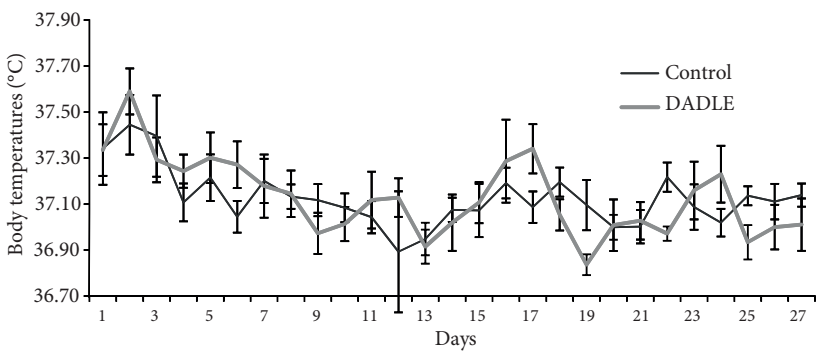

Figure 1. The daily mean body temperatures of the DADLE treatment and control groups. (Standard errors of the daily mean values are expressed as error bars.)

The coagulation parameters (APTT, PT, TT) are shown in Table 1. The activated partial thromboplastin time and prothrombin time were not altered by DADLE treatment. The thrombin time of the DADLE treated group was significantly longer than that of the control group.

Although there was a small tendency for a decrease in WBC count of the DADLE treated group compared to control animals (Figure 2), this numerical decrease was not statistically significant.

DADLE did not cause any statistically significant change in AA level in plasma (Figure 3) or TAC in plasma and brain (Table 2). Consistently the MDA levels in both groups were not significantly different (Table 3).

Discussion

To the best of our knowledge, the present study is the first investigating the effects of long-term continuous infusion of DADLE in a non-hibernating species. In this study, 28 days of continuous, subcutaneous perfusion of 0.5% DADLE solution at a rate of 2.5 μL/h did not cause any statistically significant change in body temperature (Figure 1). Vybíral and Janský (21) found short-lasting hypothermia when DADLE was injected directly into the hypothalamus of rats. They did not observe any effects when applying it at a lower dose in the same way or at a 10-fold dose intravenously. Tsao et al. (13) failed to detect any change in body temperature of mice when they applied DADLE intraperitoneally (IP). Briefly, the hypothermic effect of DADLE, observed dose-dependently after the administrations into the central nervous system, cannot be seen in systemic applications. It has been suggested that the permeation of DADLE across the blood brain barrier (BBB) is low due to its unfavorable physiochemical properties (22). The limited BBB permeability of DADLE can explain the ineffectiveness of systemic administration on body temperature in the present study.

To the best of our knowledge, there is no previous study investigating the effects of DADLE on coagulation related parameters. In this study while TT level increased ($P < 0.05$) in the DADLE treated group, there was no statistically significant difference between the groups in terms of APTT

Table 1. Coagulation parameters. (n = 24 per group) APTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time.

	Control	DADLE
APTT (s)	10.00 ± 1.3	10.23 ± 0.7
PT (s)	14.76 ± 0.5	15.27 ± 1.3
TT (s)	22.71 ± 1.7	47.90 ± 4.0*

Values are expressed in seconds, as means ± standard error of the mean.

* Difference between groups is statistically significant ($P < 0.05$)

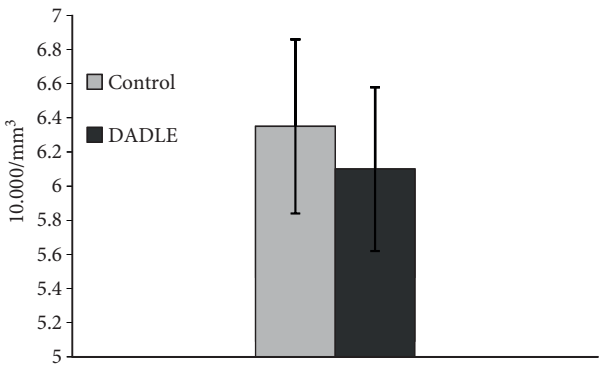

Figure 2. White blood cell (WBC) counts of the DADLE treatment and control groups. (Values are expressed as means and standard errors of the mean are shown as error bars. n = 24 per group.)

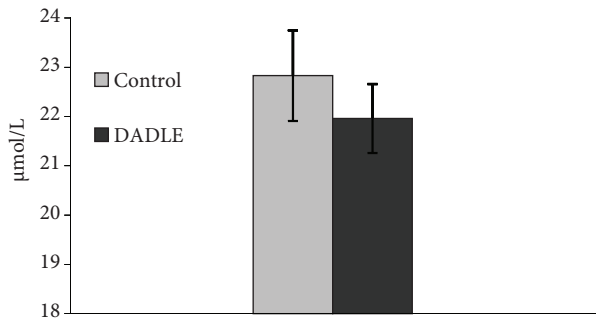

Figure 3. Plasma ascorbic acid (AA) levels of the DADLE treatment and control groups. (Values are expressed as means and standard errors of the mean are shown as error bars. n = 24 per group.)

Table 2. Total antioxidant capacity in plasma and brain (n = 24 per group).

	Control	DADLE
Plasma (mmol trolox equiv./L)	0.763 ± 0.02	0.745 ± 0.01
Brain (mmol trolox equiv./ mg protein)	11.992 ± 0.18	11.810 ± 0.10

Values are expressed as means ± standard error of the mean.

Table 3. Malondialdehyde (MDA) levels in plasma and brain (n = 24 per group).

	Control	DADLE
Plasma (nmol/mL)	10.415 ± 0.32	10.967 ± 0.22
Brain (nmol/mg protein)	0.7999 ± 0.04	0.8262 ± 0.05

Values are expressed as means ± standard error of the mean.

or PT (Table 1). Hypocoagulation, which is an important component of neuroprotective adaptation seen in hibernation, is attributed to increased levels of α-2 macroglobulin (α-2M) (23). α-2M binds factor Xa in the coagulation cascade and inhibits this activated factor at the intersection point of the intrinsic and extrinsic coagulation pathways and it also binds thrombin (24). In this study, increased TT, which assesses the activity of these 2 important components of the coagulation cascade, may be explained by the possibility of increased α-2M level in the experimental group animals.

Although there are some studies examining the immunomodulating effects of DADLE and other opioid agonists (25), the effects of systemic DADLE application on the leukogram has not been investigated thoroughly. In this study it was found that the leukocyte count of experimental group animals had a tendency to decrease compared to that of the control group, but this is not statistically significant (Figure 2). Rahim et al. (26) found that applying delta opioid agonists using osmotic pumps has an immune suppressive effect. Although the tendency for a decrease in WBCs in the experimental group was not statistically significant, this numerical decrease may be the result of increased leukocyte margination similar to the one seen in hibernation (6).

A few studies investigated the effects of DADLE on antioxidant status and oxidative stress. In one of these, Tsao et al. (13) found that intraperitoneally applied DADLE was neuroprotective in the central nervous system and attributed this protective effect of DADLE to both activation of delta opioid receptors and its free radical scavenging property in vitro. It has been demonstrated that intravenously administered DADLE protects the liver against IR injury by reduction of lipid peroxidation (12). More recently, Yang et al. (16) reported that systemically applied DADLE attenuates oxidative injury in the brain by enhancing antioxidant ability via DOR activation. In the present study, subcutaneous infusion of DADLE did not cause any significant alteration in parameters related to the antioxidant defense system (AA, TAC, MDA). This ineffectiveness could have been due to the low dose administered in our study, and the free radical scavenging property of DADLE might not have been sufficient to reveal any antioxidant activity in this low dose. Moreover, Tao et al. (27) reported that chronic DADLE treatment caused a decrease in DOR density in the rat brain. In this context, it seems possible that the chronic continuous infusion of DADLE in the present study might have similarly decreased the delta-opioid receptor density, and, therefore, DADLE treatment might not have caused any significant effect.

In conclusion, although DADLE was found to be a neuroprotective agent and had significant tissue protective effects in several studies and multiorgan preservation experiments, in this study subcutaneous infusion of 0.5% DADLE solution at the rate of 2.5 μL/h continuously for 28 days did not have any positive effects in rats concerning the antioxidant defense system and some additionally parameters related to the neuroprotective adaptation of hibernation.

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