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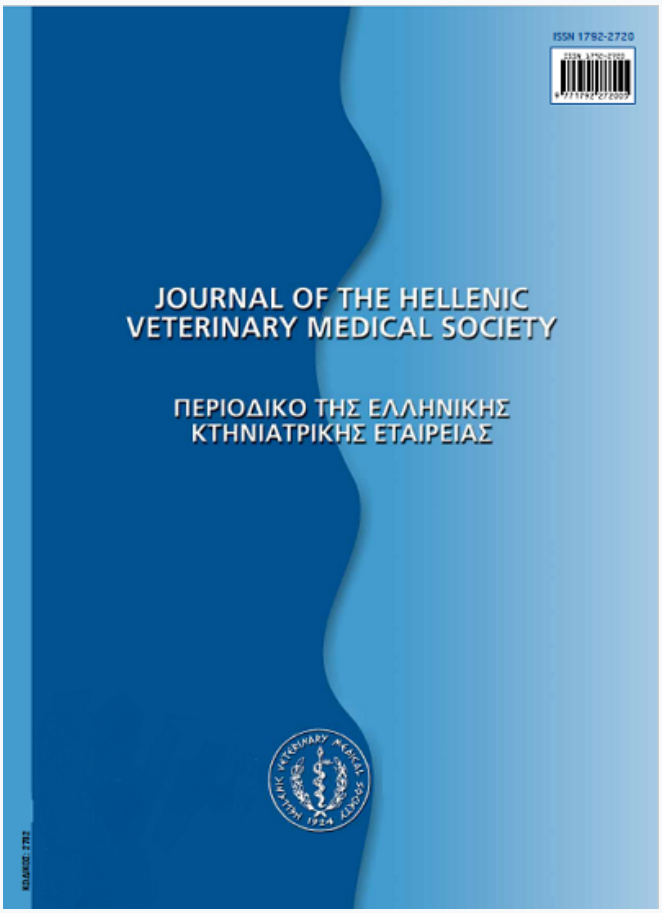

Evidence for the presence of morphine like substance and μ opiate receptor expression in Dicrocoelium dendriticum (Trematoda: Dicrocoeliidae)

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Evidence for the presence of morphine like substance and μ opiate receptor expression in *Dicrocoelium dendriticum* (Trematoda: Dicrocoeliidae)

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ABSTRACT. Parasites for living in host's body, evade from host's immune system using variant methods such as production of some molecules with immunosuppressive properties. Of these immunosuppression molecules are morphine and morphine like substances. It can effect on innate and adaptive immunity and also humoral and cellular immunity. The past researches were approved the existence of morphine in the brain and adrenal of beef and in brain of mammalian and also it has been demonstrated as an immune modulator molecule in some invertebrates, some of the nematodes and also a trematode ***Schistosoma mansoni***, But there is no document for morphine evidence in trematode ***Dicrocoelium dendriticum.*** Infection with this trematode has much importance either in health of ruminant and economic loos in the meat industry. In addition, this trematode is a zoonosis that there is no proper treatment for it in contrast with the other liver fluke, ***Fasciola hepatica***. We assume that living for a long time in the bile ducts of host is related to morphine like substance as an immune regulatory molecule. We use RP-HPLC method for detection morphine in ***D. dendriticum*** and also using reverse transcription polymerase chain reaction (RT-PCR) for μ opioid receptor finding. According to the results ***D. dendriticum*** has endogenous morphine like material and also the existence of opioid μ receptor in this trematode was approved. Living in host bile duct for a long time without sever immunopathologycal impact, suggested that probably this worm use of morphine like material as an immunoregulatory molecule and also for escaping from host immune system.

Key words*: *Dicrocoelium dendriticum, Morphine, μ receptore, HPLC, RT-PCR

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INTRODUCTION

Parasites are the organisms with advanced mechanisms especially when they infected a host, and their controlling seems difficult. Parasites can live in host's body for a long time that can evade from host's immune. They can use variant methods for escaping from immune system: 1- choosing an inaccessible sites that immune system cannot find them, 2: changing or covering the surface antigens, 3: destroying the immune molecules such as antibody digestion and reduction of MHC expression, 4: immune modulation by polyclonal activation of B cell and T cell or by inducing some molecules with immunosuppressive properties (Jacobs et al., 2016). According to the previous researches one of the molecules that has such properties is morphine (Laurent et al., 2000; Pryor and Elizee 2000; Zhu et al., 2002; Pryor et al., 2005). Morphine, an alkaloid opiate, use in medicine for pain killing. It can bind with pain receptors in CNS, distruption in release of neurotransmitters (Szkutnik-Fiedler et al., 2011) and lead to immunosuppression especially by effect on cellular immunity (Carr and Serou 1995). Opioids bind with opioid receptors. These receptors are coupled with seven transmembrane G Protein. They can sense out of the cells and active signals in cells for cell responses. Classical opioid receptors have three sub types: μ,δ,κ . These receptors appear not only on very kind of cells and tissues which are engaged in sense of pain in CNS, but also expressed by immune cells such as macrophage, T-cells, B-cells and CD4+. For the first time these have been proven by Chuang et al. 1995 by reverse transcription polymerase chain reaction method (RT-PCR). Morphine binds to μ opioid receptor preferably and induces analgesia in two pathways: indirectly through hypothalamic-pituitary-adrenal axis (HPA axis) with corticosteroids increased of adrenal; and directly through modulation the function of lymphocytes and the others immune cells (**Freier and Fuchs** 1994; Ninković and Roy 2013). Consequently it will effect on innate and adaptive immunity and also humoral and cellular immunity. The past researches were approved the existence of morphine in brain and adrenal of beef (Goldstein et al., 1985) and in brain of mammalian (Weitz et al., 1986), and also it has been demonstrated as an immune modulator molecule in some invertebrates such as ***Mytilus edulis*** (Stefano et al., 1993), ***Planorbarius*** snail (Sonetti et al., 1999) and some of the nematodes ***Ascaris suum***, ***Drancunculus medinensis*** and ***Trichinella spiralis*** and also a trematode ***Schistosoma mansoni*** (Pearce and Sher 1987; Leung et al., 1995). But there is no available research on liver trematode, ***Dicrocoelium dendriticum*** . ***D. dendriticum***, a small or medium size trematode, live in the bile and pancreatic ducts of ruminants, birds and also human .This liver fluke has a complex life cycle with two intermediate hosts: a land snail and an ant. Human infection occurs by ingestion of the second intermediate host. However ingestion a lot of infected ant is unusual and by accident so dicrocoeliasis in human occur rarely. ***D. dendriticum*** attach by its suckers lead to some pathological changes but in heavy infection they can cause to cirrhosis. Their eggs are very resistance in environment even in cold condition so it can be one of the important factors in distribution of this parasite (Jacobs et al., 2016). Infection with this trematode has much importance either in heath of ruminant and economic losses in meat industry. In addition this trematode is a zoonosis that there is no proper treatment for it in contrast with the other liver fluke, ***Fasciola hepatica*** (Jacobs et al., 2016). We assume that living for a long time in the bile ducts of host is related to morphine like substance as an immune regulatory molecule. And there is one another important reason as a hypotheses; in a new investigation in 2015, researchers have found that if hepatocellular carcinoma cell (HCC) lines getting exposed to somatic antigens of ***D. dendriticum*** (which prepared from homogenized worm) for a long time and with efficient concentration it will lead to significant reduce in their proliferation (Pepe et al., 2015), and maybe it is in relation with the other investigation about effect of morphine on the regulation of cancer cell growth (Gach et al., 2011). In the other words probably morphine was extracted together with the somatic antigens of ***D. dendriticum*** and perhaps the anti-proliferation effect of the somatic antigens of this trematode is not unrelated to the morphine. The same as the other related investigation we used HPLC method for detection morphine in ***D. dendriticum*** and also reverse transcription polymerase chain reaction (RT-PCR) for μ opioid receptor finding.

MATERIAL AND METHODS

Sample collection

The infected livers were collected from slaughterhouse of Urmia, North East of Iran, referred to Parasitology laboratory of Urmia University. Bile ducts were opened and ***D. dendriticum*** was collected. They were washed in PBS, and transferred to liquid

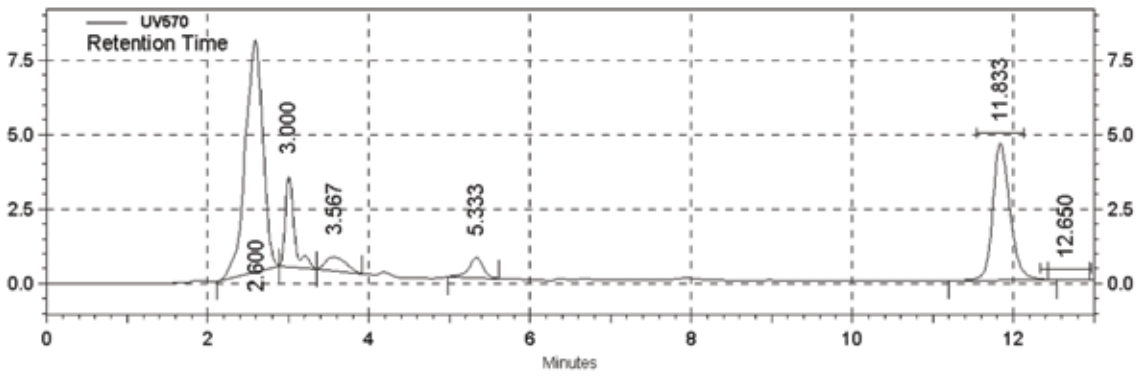
nitrogen for RT-PCR and some of them were kept in freezer -18 until HPLC analyses.

Sample preparing for HPLC

In order to analyze the worm, it must be prepared. The worms were washed in PBS after defrost, then they crushed and weighted (>0.1g), and became homogenized in 1N HCl (1ml /0.1g). Adding 5 ml solution of chloroform/isopropanol (9:1) samples were centrifuged twice (3500 rpm for 15 min). It made 3 layers and morphine is in supernatant that transferred to new tube and centrifuged (9000 rpm for 15 min). C-18 cartridges were used as a stationary phase. Methanol, HPLC water and phosphate buffer (pH 6) were used to balance the cartridges. Centrifuged samples were poured in cartridges and then washed with phosphate buffer (pH 4.5) and again washed with methanol. Dichloromethane/isopropyl/ethylacetate solution (4:12:18) were used as an elution buffer for morphine. Eluted samples were gathered by vacuum pump and dried overnight in 40°C and dissolved in HPLC mobile phase (water and acetonitrile 90:10)(v/v) containing 0.012 M phosphate buffer (pH 4). This solution were filtered by filter 0.2 micrometer for it clearance. Finally it was injected (Goumon et al., 2000).

HPLC analyzer was Knauer K-1001 pump and an Eorospher 100-5 C18 column with UV detector in 230 nm and flow rate of 0.6 mL/min and as a stationary phase Sep-Pak plus C-18 cartridges (Chromabond, Germany) were used. The method of HPLC was revers phase HPLC (RP-HPLC).

Fifure 1. Chromatogram of ***D.dendriticum*** in retention time 2.6 min (the highest peak show the morphine peaks in chromatogram). The column eluent was monitored at 230 nm, Flow rate of 0.6 mL/min. Mobile phase: 90:10 (v/v) water- acetonitrile containing 0.012 M phosphate buffer (pH 4).



μ opioid receptor gene expression

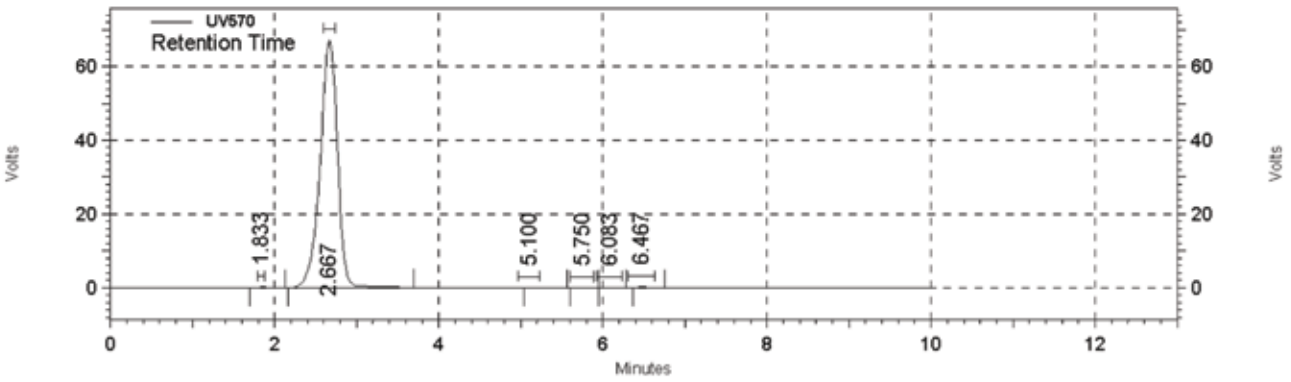
Total RNA extraction

Total body section of helminthes and also ganglions of ***Mytilus*** leech as a positive control were crash by liquid nitrogen adding lysis buffer (500μl), vortexed vigorously and centrifuged (10 min 10000g). The supeμrnant was transferred in a fresh tube and then isopropanol (300 μl) was added, vortexed and centrifuged (30 sec. 10000g). Next the mixture was washed with primary and secondary washing buffer (500 μl). Finally 50 μl of elution buffer was added, centrifuged and total RNA collected. RNA concentration and purity were determined by Nano drop 2000c spectrophotometer (Thermo scientific) and agarose gel 1%.

RT-PCR

Total RNA was extracted and in order to synthetize cDNA we used QIAGEN Onestep RT-PCR Kit (Cat No: 210212). To do so 9 μl distilled water, 5 μl buffer (5x), 1 μl dNTPs, 2 μl of each forward and reverse primers for detecting the μ receptor and 1 μl of reverse transcriptase enzyme added in a microtube and finally 5 μl of extracted RNA were added, placed in termocycler for 30 min in 50 °, 15 min in 95°,(1 min95°, 1 min 59°, 1 min 72°) for 40 cycles and 10 min at 72°C for final extension cycle. The product was run on agarose 1% gel and SYBR Green for DNA staining.

Figure 2. Chromatogram of standard morphine in retention time 2.66 min (the highest peak show the morphine peaks in chromatogram). Running conditions;The column eluent was monitored at 230 nm, Flow rate of 0.6 mL/min. Mobile phase: 90:10 (v/v) water- acetonitrile containing 0.012 M phosphate buffer (pH 4).



Total RNA purification kit (Jena bioscience), one step RT-PCR kit (Qiagen) and DNA ladder were obtained from Sinaclon Bioscience. Agarose and other reagents were purchased from Sigma-Aldrich.

The μ specific primers were: (M1: 5′- GGTACTGGGAAAACCTGCTGAAGATCTGTG-3′) (M4: 5′-GGTCTCTAGTGTTCTGACGAATTCGAGTGG-3′) the length of amplified fragment was 441 bp. This fragment corresponding to the third extracellular loop of the receptor μ.

RESULTS:

Detection of morphine like substance:

The existence of morphine- like substance in ***D. dendriticum*** was proved by, RP- HPLC and its chromatogram is shown in Fig. 1. We used the standard morphine for gaining the control curve and the chromatogram of standard morphine is shown in Fig. 2. The average of concentration of morphine was 1.7 μg/g (1700 ng/g) worm wet weight. As it is clear these peaks are similar and have the same retention times so it can suggest the similarity between these two compounds chemically.

μ opioid receptor:

The ratio of 260/280 was 2.6 and it is show the good concentration of RNA. To amplify the μ opiate receptor in leech and ***D. dendriticum***, the RT-PCR was used (Fig 3). The expected sizes (441 bp) were observed for both of them.

DISCUSSION:

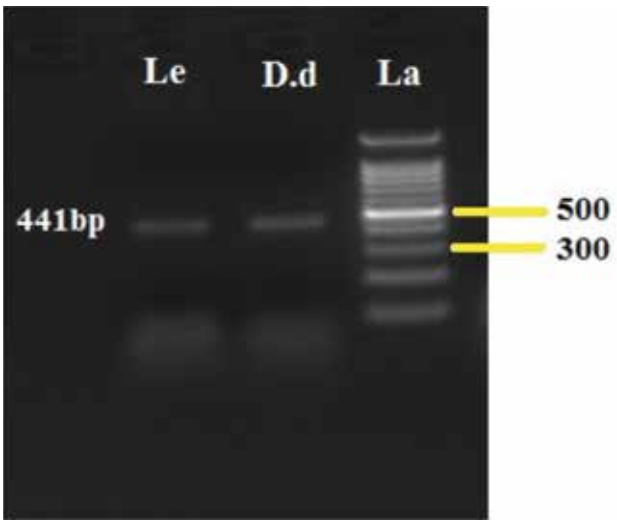
According to the results, ***D. dendriticum*** has endogenous morphine that determined by RP-HPLC and also we find out that there is opioid μ receptor in this trematode and it shows this worm can use the morphine. Morphine is an alkaloid that presents in the poppy plant. This water soluble alkaloid has 2 main metabolites in human body: morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). Morphine is metabolized in liver kidney and brain (Christrup 1997). Opioids such as morphine by binding to opioid receptors on central nervous system cells and impose an analgesia effect. However opioid receptors are distributed in other cells and tissues such as the cells of the immune system and it indicated the other effects of opioids. Morphine can change the biological response via effect on the B-cells and T-cells and produce nonspecific antibody (Carr and Serou 1995). It can influence on promoter and suppress the encoding gene of interferon alpha, a cytokine of the innate immune system, and consequently reduce the function of natural killer cells and down regulation of MHC expression (Wan et al., 2008; Owen et al., 2009). Changing in activity of immune cell effect on the production of T-helper 1 and 2 and therefore their balance will disturbed and cannot do the specific response for humoral or cellular response (Mosmann and Sad 1996).

The previous researchers indicated the existence of morphine in addition to the other opioids, in mammalian tissue like as brain, adrenal and hypothalamus (Goldstein et al., 1985; Weitz et al., 1986) and also

morphine-like compound in urine and central nervous system of the other species (Oka et al., 1985). Spector and his colleagues reported the existence of morphine in skin of a vertebrate, a toad by HPLC (Oka et al., 1985), and in 1981 it was determine that following the electrical stimulation in guinea pig ileum, morphine – like compound can prevent the contraction of it. (Oka et al., 1985). Stephano reported morphine- like substance in ***Mytilus eduslis*** by HPLC and radio immune assay and show that in stress condition the measure of morphine- like substance increase the same as human (Stefano et al., 1993) also μ receptors in this invertebrate was detected by RT-PCR (Cadet and Stefano 1999). The morphine existence in nervous and immune tissue of a snail (***Planorbarius corneus***) was determined by HPLC with electrochemical detection (Sonetti et al., 1999). Leung et al in 1995 for the first time reported the existence of morphine- like molecules in ***S. mansoni*** by HPLC and available antibody. They said that this molecule can mimic authentic morphine for immunosuppressing and escape from host immunity (Leung et al., 1995; Pryor and Elizee 2000).

Pryor et al. in 2000 had done an investigation on a nematode: ***T. spiralis***, a trematode: ***S. mansoni*** and a leech: ***Theromyzon tessulatum*** and found the morphine- like substance beside some others peptides including adreno corticotropin and beta-endorphin, in this invertbrates. They emphasized that morphine production is a mechanism for immune evasion in these species they use HPLC the same as the others (Pryor and Elizee 2000). In the same year, Goumon et al. did the similar investigation on ***Ascaris suum*** and detected the morphine- like substance by HPLC and gas chromatography/mass spectrometry (GC mass) method with the mean concentration of 1168±278 ng/gwet weight, but they couldn't showed the existence of μ opioid receptor in this nematode (Goumon et al., 2000). There is another similar report two years later; the existence of opiate alkaloid morphine and also morphine 6-glucuronide (the active metabolite of morphine) in nematode ***D. medinensis*** and also trematode ***S. mansoni*** as a molecule for immunosuppressive of host but they used the other methods except HPLC for it, double quadrupole/orthogonal-acceleration, time-of-flight mass spectrometry and nano-electrospray-ionization (Zhu et al., 2002). The concentration of morphine in ***D. medinensis*** and ***S.mansoni*** were: 11.43(4.57) and 6.24 (2.83)ng/g wet weight respectedly (Pryor, Henry et al.2005) For the first time, in the present study, except to indicate the morphine like substance in ***D.dendriticum*** by RP-HPLC (1700 ng/g wet weight) we could amplify the mu opioid receptor gene. However the difference between the morphine concentration in ***D. dendriticum*** and the others can be due to different preparing methods and also different condition of worm storage until start the trial. The presence of μ receptor has reported in ***Mytilu sedulis*** in 1999. They use of a fragment of human μ opioid gene as a primer and can amplified it. Also they show 95% similarity between this gene in leech and human (Cadet and Stefano 1999), but there is a study which is not in accordance with the present study, Gumen and Colleague couldn't amplified the μ opioid gene in ***A. suum*** (2000). The results of the present study indicate the present of morphine like material in ***D. dendriticum***. Because of its living in host bile duct for a long time and without sever immunopathologycal impact; the authors speculate this worm use of morphine as an immunoregulatory molecule for escaping from host immune system.

Figure 3. Identification of mu opioid receptor. The first line (right) is ladder, the second is μ opioid receptor transcript in ***D. dendriticum*** and the last is; μ opioid receptor transcript in leech ganglia. (Expected size: 441bp).



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