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Immunohistochemical Assessment of Inflammation and Regeneration in Morphine-Dependent Rat Brain

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Original Article

Abstract

Background: Opioids are amongst the most common abused drugs. Pathologic studies on opioid abuse are limited since the evaluation of inflammation and regeneration in brain tissue is not as simple as other tissues of the body. Thus, the present study aimed to determine the relationship between the dependence on morphine and inflammatory and regenerative processes.

Methods: In this experimental study, 48 male wistar rats were divided into 6 groups. The dependent groups (3 groups) received 0.4 mg/ml morphine in drinking water for 7, 28, and 56 days. The control groups (3 groups) received sucrose solution in drinking water for the same period. The histopathological studies of the brain sample were done. The slides were stained by hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining method. The areas of brain were evaluated in terms of lymphocytic infiltration and glial scar.

Findings: A significant difference was observed in the mean number of cells in the glial scar of the dependent group 3 (dependent for 56 days) among the control group (P = 0.040). Further, a significant relationship was reported between the increased duration of morphine use and the number of created scar glial cells. Furthermore, a significant increase in the number of astrocytes was observed in the affected areas.

Conclusion: After long-term use, opioids can result in increased number of astrocytes and creating glial scar centers in the affected areas in response to the inflammation.

Keywords: Brain; Immunohistochemistry; Morphine; Rats

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Introduction

Opioids such as morphine and heroin are considered as common abused substances.[1] According to the international reports, Iranian adults have the highest worldwide rate of opioid addiction as high as 2.8%.[2] However, morphine is the most common analgesic agent used for moderate to severe pains.[1] A large number of studies have been conducted on different organs indicating tissue injury and induced necrosis by inflammatory phenomenon and fibrosis. Neurological system may involve the most severity since it is regarded as the first affected organ.[3]

So far, different studies have empahasized the adverse effects of opioids on the brain, leading to some biochemical alterations and radiological changes.[4-6] However, pathological studies on opioids abuse have been less considered since the evaluation of inflammation and regeneration in brain tissue is not as simple as other body tissues. Obviously, brain tissue exposed to morphine is different from normal brain tissue due to metabolic, receptor, genetic, and environmental factors.[7,8] In addition, different neuronal effects have caused the long-term use of morphine leading to neuronal injury.[4] The number of documents indicating persistent cognitive disorders in patients using opioids is increasing.[6] Based on animal studies, chronic morphine exposure results in creating some significant neurological alterations although they failed to recognize the small effects on brain tissue.[9]

Chronic neuroinflammation usually occurs due to brain infections, autoimmune diseases, brain traumatic injuries, and toxic metabolites.[10] The last one is related to responsible etiology in morphine intoxication. In chronic inflammation site in the brain, the glial cells are aggregated and the B and T lymphocytes are observed in inflamed region if blood-brain barrier is injured.[11] The use of immunohistochemistry (IHC) staining is crucial since glial cells' nuclei, especially oligodendrocytes, are similar to lymphocytes[12] for confidence from lymphocyte presence and perfect estimation of inflammatory cells. Leukocyte common antigen (LCA, CD45) is a common confident marker for distinguishing inflammatory markers.[13] However, tissue regeneration is different and astrocyte proliferation is observed in injury location as glial scar.[14] Routine staining cannot differentiate between astrocytes and glial cells, and glial fibrillary acidic protein (GFAP) has been used to demonstrate the glial scar in a large number of studies.[15-17] In addition, GFAP is one of the five most common medium cytoplasmic filaments (MW48000-52000) which are observed in active normal cells and neoplastic astrocytes.[13]

Preventing brain tissue damage by inflammation inhibitor mechanisms is possible if the damage related to brain tissue is due to induced inflammation and healing while using opioid drugs chronically. Thus, the present study aimed to determine the relationship between morphine dependence with inflammatory and regenerative processes.

Methods

In this experimental study, the paraffin blocks from brain tissues of 48 16-month-old male Wistar rats with mean weight of 230 g were evaluated. Animals were stored according to food and biological conditions of European Community Guidelines. The study was conducted in six groups of 8 rats, among which 3 received water-soluble morphine powder for 7, 28, and 56 days, respectively. Regarding three control groups, the 0.3% sucrose solution was used for 7, 28, and 56 days, respectively.

The dependence procedure was as follows: the 7-day group with 0.4 mg/ml morphine was exposed to drinking water for 7 days. In the 28-day group, 0.1 mg/ml morphine was placed in drinking water for the first 48 hours by increasing 0.1 mg/ml per 48 hours, which raised to 0.3 mg/ml in the sixth day continuing with 0.4 mg/ml up to 28th day. In the third group, the initiation was similar to the second group and continued with maximal dose of 0.4 mg/ml up to the end of 56th day. In order to have better tolerance of morphine taste, the sucrose was added to drinking water in all groups (3 g per liter). Since a majority of morphine effects was relieved by naloxone, it was used to establish the dependence. 10% of the rats were randomly selected in the second and third groups and 2 mg/kg naloxane was injected and assessed for withdrawal symptoms at 21st day of the study including head and limb tremor, jerking, diarrhea, agitation, eyelid ptosis, body stretching, and teeth rubbing. The morphine dependence was enough when there were four symptoms or more during 20 minutes.

In addition, the animals were anesthetized with ether and killed with formalin heart injection at the end of each period. Then, the brain was removed carefully after craniotomy. For the purpose of blindness, all samples attained code by generating random numbers and were referred for histopathological assessment. In order to provide a similar sampling location, the specimens were prepared with 5 mm thickness from 3.2-3.7 mm after bregma for frontal region and 3.2 mm before bregma for parietal cortex and hippocampus. The code was received with blind assessment after the slides were stained with IHC method.

Regarding the fixation, the samples were inserted in 10% formalin solution and the solution was exchanged once. The tissue was processed with Duplex tissue processor (Shandon-Elliot Ltd., USA). Then, dehydration was done during five stages including one-hour 50° alcohol, one-hour 50° alcohol, one-hour 70° alcohol, one-hour 90° alcohol, one-hour 100° alcohol, and one-hour 100° alcohol. Clearing was conducted during two stages of one hour, each one with xylol. Further, the embedding was done with paraffin 1.5-hour and two-hour stages. Furthermore, blocking was conducted in the melted paraffin. Then, they were transferred to freezed store. Among the blocks, three 4-microne cuts were attained on glass slides. In addition, the paraffin was removed from the slides. Intracellular peroxidase activity was arrested with 1% H_2O_2 and 1% solution in phosphate-buffered saline (PBS) for 30 minutes. In the next stage, the antigen was recovered by incubating the slides in 10 mmol citrate sodium in 100 °C for 30 minutes in microwave device.

Staining was conducted by hematoxylin and eosin (H&E) stain. The first and second xylol stage lasted for 3-5 minutes. Furthermore, one-hour 50° alcohol, one-hour 50° alcohol, one-hour 70° alcohol, one-hour 90° alcohol, one-hour 100° alcohol, and one-hour 100° alcohol were used. Distilled water was used several times. Then, hematoxylin was used for 5-15 minutes. Additionally, pipe water was used to wash and alcohol-acid was used with intermittent washing by pipe water. The lithium carbonate was used up to blue staining of the cut. The eosin was used for 1-3 minutes after washing by water pipe for 15 minutes. In the next stage, alcohols with different degrees from 96 to100 degrees were used for several times. Finally, xylol was used for three times within three minutes.

Polyclonal antibody against GFAP and CD45 (Dako Corp., Carpinteria, California, USA) was used nighttime in 4 °C. Then, the slides were incubated with PBS after washing for the purpose of biotinylation with secondary immunoglobulin G (IgG) antibody for 30 seconds in room temperature. In addition, the Vectastain Elite ABC-peroxidase kit (Vector Laboratories, Burlingame, California, USA) was used for reticular staining. Finally, the slides were mounted with Gel/Mount (Biomeda Corp., Foster City, California, USA).

Additionally, the slides were assessed by Olympus CH30 microscope through amplifying for 400 times. Then, the ocular microscope was added to calculate the pathological criteria such as glial scar diameter. The pathological criteria were lymphocyte infiltration in three different samples of brain tissue including frontal cortex, parietal cortex, and hippocampus. The count was assessed with power 400 and the mean was recorded when there were lymphocytes. Glial scars as astrocyte aggregation in the injured regions were evaluated in three different parts.

The data were analyzed by SPSS software (version 12, SPSS Inc., Chicago, IL, USA). Mann-Whitney-U, independent samples t-test, one-way analysis of variance (ANOVA), and Bonferroni post-hoc tests were used to comapre the means between three different samples of brain tissue including frontal cortex, parietal cortex, and hippocampus and the significance level was considered to be 0.050.

Results

As shown in table 1, no significant difference was observed between the glial scars across the groups ($P > 0.050$).

Table 1. Glial scars across three groups

Groups		Count (percent)	P
1	Dependent	2 (25.0)	> 0.999
	Control	2 (25.0)	
2	Dependent	4 (50.0)	0.500
	Control	3 (37.5)	
3	Dependent	5 (62.5)	0.300
	Control	2 (25.0)	

Despite a lack of difference in the number of glial scars ($P > 0.050$), a significant difference was

observed between the dependent and control subgroups in group 3 (P = 0.040), indicating the role of longer time in higher count of glial scars. In addition, as illustrated in figure 1, no statistically significant difference was observed between the three groups (P = 0.080).

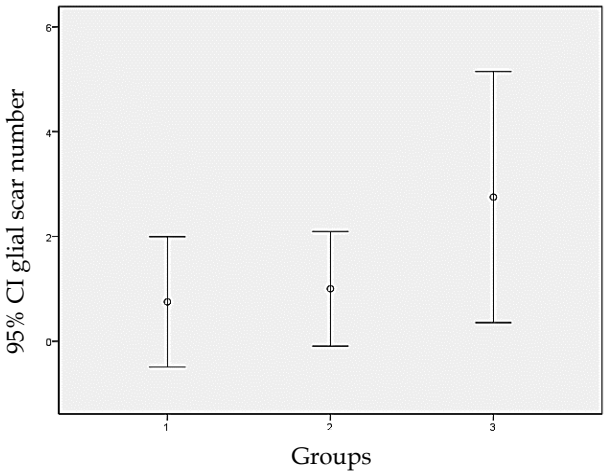

Figure 1. Number of glial scars across the groups

As shown in table 2, the mean diameters of glial scars were not significantly different between the dependent and control groups and across the dependent groups (P > 0.050). Histologic finding are shown in figure 2.

Table 2. Glial scar diameters across three groups

Group		Mean ± SD	P
1	Dependent	30.0 ± 7.0	0.870
	Control	35.0 ± 3.3	
2	Dependent	32.5 ± 6.4	0.720
	Control	27.5 ± 3.5	
3	Dependent	32.0 ± 5.7	0.650
	Control	30.0 ± 3.2	

SD: Standard deviation

Discussion

This study showed that long-term use of opioids in rats would lead to damage in brain tissue as response to inflammation. Based on the results of different studies, brain aging can result in altering biochemical and signal transduction. In addition, the brain tissue affected by morphine is significantly different from the normal tissues.[7] In another study on elderly patients, Hamzaee Moghaddam et al.[18] reported asignificant difference in Alzheimer incidence between addict and non-addict groups, possibly due to higher susceptibility of elderly exposed to opioids.

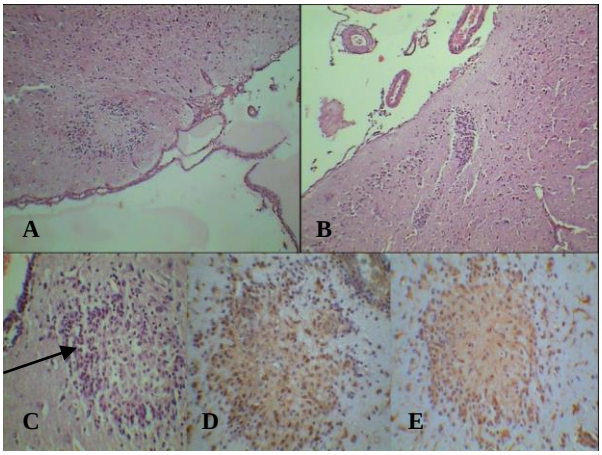

Figure 2. A: Histology of rat brain; B: Hematoxylin and eosin (H&E) staining (magnification x100 in both); C: H&E staining (magnification x400), arrows show glial scar; D and E: Immunohistochemistry (IHC) staining for glial fibrillary acidic protein (GFAP) (magnification x400), astrocytes show positive reaction in glial scar.

Further, Malekpour Afshar et al.[19] investigated brain histopathological alterations in addict aged rats and reported a significant neuronal cell reduction in frontal and temporal regions among opium-addicted rats. Thus, neurotoxic morphine effects lead to neuronal apoptosis. Furthermore, a significant reduction occurred in hippocampal neurons involved in memory functions. In addition, Lyoo et al.[20] reported a decrease in prefrontal and temporal gray matter density in opiate dependence.

As it was already mentioned, few studies have been conducted on the pathological effects of opioid abuse. Regarding the lack of similar studies in this area, the present study was performed to assess the inflammation process as a trigger for tissue injury. In this regard, the IHC staining was used for confidence from the presence or absence of lymphocytes and definite measurement of inflammatory cells.

The regeneration markers in brain tissue are completely different and the astrocyte proliferation is available in injury site called "glial scar" instead of fibroblasts.[14] Routine staining is not able to differentiate between astrocytes and glial cells, and GFAP was used to demonstrate the glial scar in a large number of studies.[15-17]

Conclusion

Based on the results, after long-term use, opioids can damage brain tissue in the form of the increased number of astrocytes and the creation of

glial scar centers in the affected areas in response to the created inflammation. These changes are more severe in chronic substance abuse and may indicate the neurotoxic effects of morphine. Further, due to changes in the brain tissue at different levels with aging,[21] the present study was conducted on the brain of old rats and the results on frontal, parietal, and hippocampus lobes of the brain, which are the most sensitive brain areas[19] when faced with injury, may be related to more vulnerability of elderly patients to opioid compounds.

Conflict of Interests

The Authors have no conflict of interest.

Acknowledgements

None.

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بررسی التهاب و ترمیم در مغز موش‌های صحرایی معتاد به مورفین به روش ایمنوهیستوشیمی

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مقاله پژوهشی

چکیده

مقدمه: ترکیبات اپیوئیدی، از جمله شایع‌ترین ترکیباتی هستند که مورد سوء استفاده دارویی قرار می‌گیرند. مطالعات پاتولوژیک در زمینه سوء مصرف اپیوئیدها بسیار محدود می‌باشد. از آن‌جا که ارزیابی التهاب و ترمیم در بافت مغزی به سادگی سایر بافت‌های بدن میسر نیست، پژوهش حاضر با هدف بررسی ارتباط بین وابستگی به مورفین و روندهای التهابی و ترمیمی انجام گردید.

روش‌ها: در این مطالعه تجربی، ۴۸ موش صحرایی نر نژاد ویستار به شش گروه یکسان تقسیم شدند. گروه‌های وابسته (سه گروه) ۰/۴ میلی‌گرم بر میلی‌لیتر مورفین در آب آشامیدنی را برای ۷، ۲۸ و ۵۶ روز دریافت کردند. به گروه‌های شاهد (سه گروه) نیز محلول ساکاروز در آب آشامیدنی برای مدت مشابه داده شد. مطالعات هیستوپاتولوژیک بر روی نمونه‌های مغز صورت گرفت. اسلایدها به روش هماتوکسیلین- ائوزین (Hematoxylin and Eosin یا H&E) و ایمونوهیستوشیمی رنگ‌آمیزی گردید و از نظر ارتشاح لنفوسیتی و اسکار گلیال مورد ارزیابی قرار گرفت.

یافته‌ها: تفاوت معنی‌داری از لحاظ میانگین تعداد کانون‌های اسکار گلیال در گروه وابسته ۳ (به مدت ۵۶ روز) با گروه شاهد آن مشاهده شد (P = ۰/۰۴۰). همچنین، یافته‌هایی حاکی از ارتباط بین افزایش مدت زمان مصرف مورفین با تعداد کانون‌های اسکار گلیال ایجاد شده وجود داشت. علاوه بر این، افزایش قابل توجه تعداد سلول‌های آستروسیتی نیز در نواحی آسیب دیده قابل مشاهده بود.

نتیجه‌گیری: مواد اپیوئیدی بعد از مصرف طولانی مدت، می‌توانند منجر به افزایش سلول‌های آستروسیتی در نواحی آسیب دیده و ایجاد کانون‌های اسکار گلیال در این نواحی در پاسخ به التهاب به وجود آمده شوند.

واژگان کلیدی: مغز، ایمنوهیستوشیمی، مورفین، موش‌های صحرایی

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