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Editorial:
Brain Extracellular Space: Geometry, Matrix and Physiological Importance

What is ECS and why is it important?

Brain tissue is essentially composed of two regions: cellular elements (neurons and glial cells), and the gap between the elements, which is known as the extracellular space (ECS; Figure 1) (Sykova & Nicholson, 2008). The ECS resembles the water phase of a foam and remains a highly connected domain even though it is convoluted in shape and may form dead-space microdomains (e.g. local expansions or voids) (Hrabetova, Hrabe, & Nicholson, 2003). The width of the ECS is about 20-60 nm (Thorne & Nicholson, 2006), nevertheless in totality it occupies approximately 20% of the entire tissue volume (Sykova & Nicholson, 2008) The surprisingly large relative volume of the ECS makes it an important area for neuroscience research.

Extracellular space is the immediate external environment of brain cells. This proximity to the cell membrane makes the structure and content of the ECS important for cellular homeostasis and function. The ECS contains a fluid similar in composition to that found in the brain ventricles that maintains an ionic balance for Ca^{2+}, Na^{+}, K^{+} and Cl^{-} across the cell membrane. Such an ionic balance establishes the cellular resting potential and permits neuronal action potentials and synaptic transmission. The ECS also provides a communication channel between cells through which chemical signals travel; this is known as volume transmission (Agnati, Fuxe, Nicholson, & Sykova, 2000). Clinically, the ECS is an important route for the delivery of drugs after they have entered the brain (Wolak & Thorne, 2013).

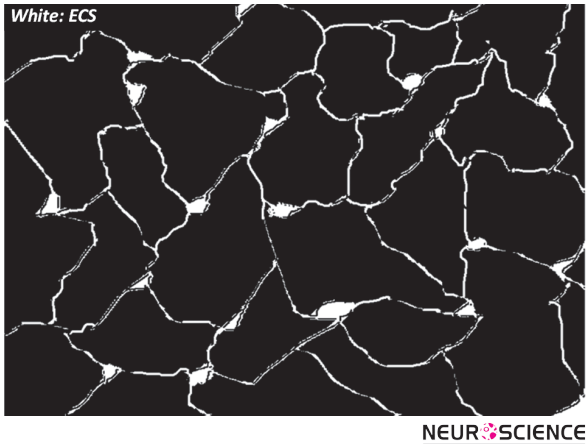

Figure 1. Schematic of brain cells and ECS. The ECS may have local expansions.

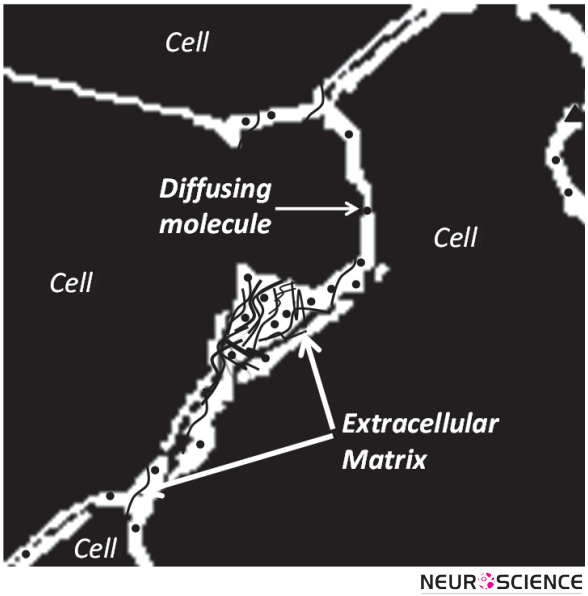

Figure 2. Schematic of the extracellular matrix as a meshwork of long-chain molecules distributed in ECS.

Besides an ionic fluid, the ECS accommodates an extracellular matrix formed from a meshwork of long-chain polymeric molecules and proteins (Figure 2). These include chondroitin sulfate, heparin sulfate and tenasin that often branch off from a hyaluronic acid backbone (Zimmermann & Dours-Zimmermann, 2008).

Molecular Diffusion in ECS

Diffusion is the dominant mechanism for transport of substances in ECS and determines both the local and global distribution of many molecules.

Both geometry of ECS and the properties of the extracellular matrix affect diffusion. The geometry of the ECS hinders free diffusion of molecules in general, while the matrix may increase local viscosity or act more specifically on molecules that undergo steric or electrostatic binding with the matrix.

Among other factors, such as the local sources or sinks of molecules, diffusion depends on the space or volume fraction accessible to the molecules and the geometry of their path through the ECS. Volume fraction of ECS, α, is defined as the dimensionless parameter:

$$\alpha = V_{ECS} / V_{Tissue} \quad (1)$$

where V_{ECS} and V_{Tissue} are the volumes of ECS and the whole tissue respectively.

Molecular diffusion in ECS is similar to that in a porous medium and surprisingly, using only the classical theoretical framework for diffusion, we are able to characterize molecular diffusion in the brain (Nicholson & Phillips, 1981; Nicholson, 2001). This enables us to use a single diffusion coefficient (D^*) to capture all the effects of the environment. Therefore D^* is called the 'effective diffusion coefficient'.

The magnitude of D^* reflects the hindrance imposed by the geometry of the path, therefore $D^* < D$, where D is the free diffusion coefficient. The dimensionless parameter tortuosity, λ, may be used to characterize the hindrance to diffusion where:

$$\lambda = \sqrt{D / D^*} \quad (2)$$

In addition to being affected by the geometry, the diffusing molecule may also interact with the matrix; this too can be incorporated into the tortuosity (Nicholson, Kamali-Zare, & Tao, 2011).

A measurement of tortuosity may be thought of as revealing properties of ECS itself (Nicholson & Sykova, 1998). When molecules are released from a source and make multiple random walks that are reflected from the multiple boundaries of the ECS, they effectively explore their microenvironment. If their collective behavior can be visualized, the local structure will 'appear'. The simulation shown in Figure 3 exemplifies this concept by tracking a number of molecules released from a point source in the ECS at the center of an initially unseen structure. Then, following the molecules in time, the shape of the structure emerges. The structure is revealed to be an ensemble of cubes where one in every eight is missing, providing a local expansion of ECS that constitutes a dead-space (Figure 3, last panel).

The ECS has been most studied in neocortex, however there have been measurements in corpus callosum, hippocampus, cerebellum, caudate nucleus and spinal cord. In the cerebellar molecular layer and in regions containing major fiber bundles, diffusion is anisotropic, being different in different axes (Rice, Okada, & Nicholson, 1993).

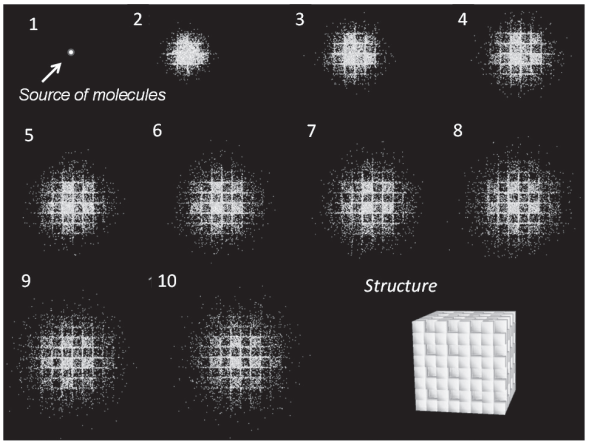

Figure 3. Projection from top of a simulation with a population of molecules released from a point source located at the center of a structure. As molecules diffuse, they make random walks and thereby reveal the structure of the local environment. The *MCell* program was used for simulation and *DReAMM* for visualization (see Modeling section).

How to Characterize ECS

Experiments provide values for volume fraction, tortuosity and some other parameters. Complementing experiments, modeling tests hypotheses about the factors that determine these parameter values. In addition, modeling establishes a solid theoretical framework for molecular diffusion in ECS. Modeling may provide a simpler alternative to experiments and sometimes may be the only way to proceed.

Experiments

The properties of the ECS have been studied with three main experimental techniques. The first is the radiotracer method in which a radio-labeled molecule, such as sucrose, is infused in a brain ventricle and its diffusion pattern is measured at later times in fixed brain tissue samples (Fenstermacher & Kaye, 1988). In the second technique, called the real-time iontophoretic (RTI) method, a small molecule, typically tetramethylammonium (TMA^+) is released from a point source and its concentration at a short distance away, measured with an ion-selective microelectrode (Nicholson & Phillips, 1981). The third method, known as integrative optical imaging (IOI), uses a fluorescently labeled macromolecule, such as dextran, or a protein molecule as a probe (Nicholson

& Tao, 1993). The RTI and IOI methods were introduced by the Nicholson laboratory and provide real-time data in small brain regions.

Modeling

Here we summarize one modeling approach based on Monte Carlo simulation using the program *MCell*, (Stiles & Bartol, 2001; Nicholson, Kamali-Zare, & Tao, 2011). *MCell* was developed at the University of Pittsburgh, Supercomputing Center and Salk Institute for Biological Studies, as a modeling tool for realistic simulation of the behavior of molecules in the complex 3D microenvironments found in biological tissue. The main advantage of using *MCell* in ECS studies is that it can represent both the ramified geometry and the molecular interactions with the extracellular matrix.

The Monte Carlo method mimics actual diffusion. A large population of 'molecules' is released from an appropriate source and the molecules execute random walks in a specified geometry. They may interact with other molecules or sites through suitable kinetic reactions. After a certain time the distribution of the molecules is analyzed. In our applications the main output of an *MCell* simulation is D^* (which is easily converted to the tortuosity). For a population of molecules released from the origin in a 3D medium, D^* is calculated using the classical equation:

$$D^* = \langle r^2 \rangle / 6t \qquad (3)$$

where r is the distance of each molecule from the source and $\langle r^2 \rangle$ represents the mean square distance of all molecules at time t after the molecules have been released.

Key Facts about ECS Derived from Experiments and Modeling

The basic quantitative parameters of ECS structure are volume fraction, α, and tortuosity, λ. Using the RTI method with TMA^+ as a small probe molecule it has been established that $\alpha = 0.2$ (this implies that the ECS occupies 20% of the brain) and $\lambda = 1.6$ (this implies that $D^* \cong 0.4\ D$). This value of tortuosity is valid for molecules that are much smaller than the width of the ECS and do not interact with the extracellular matrix. If the molecules are much larger (Thorne & Nicholson, 2006) or reversibly bind to the matrix (Hrabetova, Masri, Tao, Xiao, & Nicholson, 2009), λ may be greater than 1.6. Figure 4 summarizes the two key players in all ECS narratives: 'Geometry' and 'matrix' and emphasizes that a study of diffusion is a key to understanding ECS structure and content.

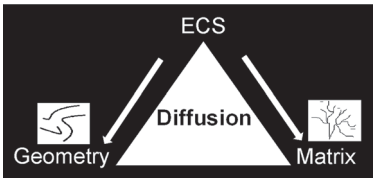

Figure 4. Schematic of major players in ECS: geometry and matrix. Diffusion can characterize both players.

Geometry of the Extracellular Space

It appears that $\lambda = 1.6$ represents a fundamental constant of the ECS and it is pertinent to ask where the value comes from. Assuming that no matrix interaction is involved it is reasonable to look to ECS geometry for the answer.

To address the role of structure, the Nicholson laboratory developed several models to explore idealized ECS geometry. The basic element of these structures is a single cube with a size similar to that of a brain cell body. In MCell simulations where many such cubes are packed together in a 3D geometry and separated from each other by a uniform ECS with a realistic width to ensure $\alpha = 0.2$, it is found that $\lambda = 1.18$ (Tao & Nicholson, 2004). Clearly, this value is much smaller than the experimentally measured value of $\lambda = 1.6$. To try to increase this low tortuosity, a number of local voids, or dead-spaces, were introduced in the cube-ensembles (Figure 5). This strategy was able to increase λ to about 1.6 because when molecules enter these local voids, they are transiently held up in the region and their diffusion time is prolonged (Tao, Tao, & Nicholson, 2005). Dead-spaces in real biological tissue may be formed by local expansions of the ECS (voids), membrane invaginations or by glial cells wrapping around neurons (Hrabetova, Hrabe, & Nicholson, 2003; Hrabetova & Nicholson, 2004).

Extracellular Matrix

In addition to the effect of the complex geometry of ECS, molecular diffusion is affected by the extracellular matrix. The matrix may react with suitable molecules through electrostatic or steric (actual binding and unbinding) interactions with the chondroitin sulfate (Hrabetova, Masri, Tao, Xiao, & Nicholson, 2009) or heparan sulfate (Thorne, Lakkaraju, Rodriguez-Boulan, & Nicholson, 2008) components of the matrix. Our current modeling studies aim to combine geometry and matrix to study how the two components interact to affect molecular diffusion (Figure 5C and 5D; Nicholson, Kamali-Zare, Tao, 2011).

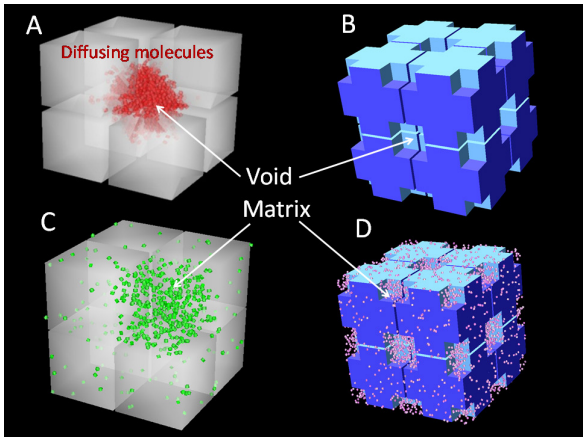

Figure 5. Geometrical elements used to construct representative geometries of the brain for *MCell* simulations. Length of each cube side = 0.6 µm and the gap between cubes equals the width of the ECS (46 nm). A) Geometry with one out of eight cubes is missing to represent a local void. B) Geometry with voids generated from cutting off the corners of each cube (length of the void = 0.15 µm). C) The same geometry as Panel A filled with uniform matrix. D) The same geometry as Panel B filled with uniform matrix. Note that these ensembles of eight cubes are replicated many times to form a medium for the simulation.

ECS Applications

Research involving ECS ranges from basic to applied. Basic research aims to answer fundamental questions about how simple mechanisms lead to complex functions. For example, it is extending the concept of the 'microdomain' from being only the small region around single channels to being a larger domain spanning the spaces surrounding groups of cells. This links molecular and cellular level events to networks of cells with subtle interactions. This perspective may help our understanding of complex diseases, such as cancer, (Vargova et al., 2003) and brings ECS research into the realm of translational research where the knowledge of basic science is applied to find innovative ways to treat diseases.

Another translational research area where ECS studies are essential is drug delivery (Wolak & Thorne, 2013). Drugs may be introduced to the brain by infusion into ventricular or spinal cavities; this offers good distribution to the targets near the cavity, but poor penetration to more distant regions. Drugs may also enter the brain via the blood supply (following oral, intramuscular or intravenous administration) but they have to pass through blood-brain-barrier, which often restricts drug candidates to small lipophilic compounds. This method also lacks targeting to specific brain regions.

Finally, drugs may be introduced via direct injection through a cannula into brain or spinal cord. This method is called convection enhanced delivery (CED) and allows focal application to the target but it is invasive and has the potential for damage (Morrison, Laske, Bobo, Oldfield, & Dedrick, 1994). In all these methods of delivery the final common path for the drug to arrive at its destination is usually diffusion through the ECS.

Conclusions

The ECS is a vital but neglected component of the cell microenvironment. The properties of the ECS affect diffusion and local concentrations of many molecules within this narrow but complex space. It is important for creating the conditions that permit neuronal electrical and chemical activity and extracellular signaling via volume transmission.

The geometry of extracellular space and interaction with matrix combine to modify the free diffusion of molecules in the brain. This gives ECS the potential to regulate diffusion of each molecule individually and dispatch them to specific targets.

Research on the ECS has a wide range of applications from addressing fundamental questions to finding innovative ways to treat diseases and deliver drugs.

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References

Agnati, L. F., Fuxe, K., Nicholson, C., Sykova, E., 2000. Volume Transmission Revisited. Progress in Brain Research, vol. 125. Amsterdam: Elsevier.

Fenstermacher, J., & Kaye, T. (1988). Drug diffusion within the brain. Annals of the New York Academy of Sciences, 531, 29-39.

Hrabetova, S., Hrabe, J. & Nicholson, C. (2003). Dead-space microdomains hinder extracellular diffusion in rat neocortex during ischemia. Journal of Neuroscience 23(23): 8351-8359.

Hrabetova, S., Masri, D., Tao, L., Xiao, F., & Nicholson, C. (2009). Calcium diffusion enhanced after cleavage of negatively charged components of brain extracellular matrix by chondroitinase ABC. Journal of Physiology, 587(16), 4029-4049.

Hrabetova, S., & Nicholson, C. (2004). Contribution of dead-space microdomains to tortuosity of brain extracellular space. Neurochemistry International 45(4): 467-477.

Morrison, P. F., Laske, D. W., Bobo, H., Oldfield, E. H., Dedrick, R. L., 1994. High-flow microinfusion: tissue penetration and pharmacodynamics. American Journal of Physiology 266, R292-R305.

Nicholson, C. (2001). Diffusion and related transport mechanisms in brain tissue. Reports on Progress in Physics, 64(7), 815-884.

Nicholson, C., Kamali-Zare, P., & Tao, L. (2011). Brain extracellular space as a diffusion barrier. Computing and Visualization in Science 14(7): 309-325.

Nicholson, C., & Phillips, J. M. (1981). Ion diffusion modified by tortuosity and volume fraction in the extracellular microenvironment of the rat cerebellum. Journal of Physiology 321: 225-257.

Nicholson, C., & Sykova, E. (1998). Extracellular space structure revealed by diffusion analysis. Trends in Neuroscience, 21(5), 207-215.

Nicholson, C., & Tao, L. (1993). Hindered diffusion of high molecular weight compounds in brain extracellular microenvironment measured with integrative optical imaging. Biophysical Journal, 65(6), 2277-2290.

Rice, M. E., Okada, Y. C., & Nicholson, C. (1993). Anisotropic and heterogeneous diffusion in the turtle cerebellum: implications for volume transmission. Journal of Neurophysiology, 70(5): 2035-2044.

Stiles, J., & Bartol, T. (2001). Monte Carlo methods for simulating realistic synaptic microphysiology using MCell. Computational Neuroscience: Realistic Modeling for Experimentalists, ed. De Schutter E. CRC Press, Boca Raton: 87-127.

Sykova, E., & Nicholson, C. (2008). Diffusion in brain extracellular space. Physiological Reviews, 88(4), 1277-1340.

Tao, L., & Nicholson, C. (2004). Maximum geometrical hindrance to diffusion in brain extracellular space surrounding uniformly spaced convex cells. Journal of Theoretical Biology, 229(1), 59-68.

Tao, A., Tao, L., & Nicholson, C. (2005). Cell cavities increase tortuosity in brain extracellular space. Journal of Theoretical Biology, 234(4), 525-536.

Thorne, R. G., Lakkaraju, A., Rodriguez-Boulan, E., & Nicholson, C. (2008). In vivo diffusion of lactoferrin in brain extracellular space is regulated by interactions with heparan sulfate. Proceedings of the National Academy of Sciences of the United States of America, 105(24), 8416-8421.

Thorne, R. G., & Nicholson, C. (2006). In vivo diffusion analysis with quantum dots and dextrans predicts the width of brain extracellular space. Proceedings of the National Academy of Sciences of the United States of America 103(14): 5567-5572.

Vargova, L., Homola, A. Zamecnik, J., Tichy, M., Benes, V., & Sykova, E. (2003). Diffusion parameters of the extracellular space in human gliomas. Glia 42(1): 77-88.

Wolak, D. J., & Thorne, R. G. (2013). Diffusion of macromolecules in the brain: implications for drug delivery. Molecular Pharmaceutics. doi: 10.1021/mp300495e.

Zimmermann, D. R. & Dours-Zimmermann, M. T. (2008). Extracellular matrix of the central nervous system: from neglect to challenge. Histochemistry and Cell Biology 130(4): 635-653.

Letter to Editor:

Does the Ability to Make a New Business Need More Risky Choices during Decisions? Evidences for the Neurocognitive Basis of Entrepreneurship

Dear Editor, As we know, individual differences play an important role in entrepreneurship ability (Zhao & Seibert, 2006). Some studies have found significant correlations between individuals' personality and entrepreneurship (White, et. al., 2006, 2007; Furnham and Nederstrom, 2010). Personality characteristics such as tolerance for risk, preference for autonomy, and innovativeness are important factors in the selection of entrepreneurs (White, et. al., 2007). Recently the study of personality as a concrete psychological concept has concentrated on brain cognitive functions (Michalski & Shackelford, 2010; Prabhakaran et. al., 2011). Hence, it has been argued that understanding entrepreneurial behaviors require understanding entrepreneurial thinking. The identification of entrepreneurship behavior requires the codification of neural base of entrepreneurial thought at a deeper level (Prabhakaran et. al., 2011). Some studies have shown that attitudes toward risk, entrepreneurial ability, preferences for autonomy, and locus of control are important in determining who starts and operates businesses (Caliendo, Fossen, & Kritikos, 2010; Zhao & Seibert, 2006).

A whole new field of neuroeconomics has made important progress in studying economic behavior at neural level (Glimcher et. al., 2009). This field has attempted to link aspects of economics such as finance (Knutson and Bossaerts, 2007), marketing (Plassmann et. al., 2007) and entrepreneurship (Krueger, 2007) with neuroscientific research methodology. Studies have identified neural mechanisms underlying the representations of value, reward, and risk, which are important factors affecting economic behavior (Platt and Huettel, 2008; Rangel et al., 2008; Schultz, 2006).

Risk-taking in some studies has been considered as a deficit in the cognitive system so that it has been linked with violence related to addiction, drug and alcohol use, and sexual risk-taking (Blum & Nelson-Mmari, 2004; Williams, Holmbeck, & Greenley, 2002; Fridberg, et. al., 2010). However, some researchers have shown that risk-taking behavior may serve some positive functions in adulthood (Dworkin, 2005; Hendry &Kloep, 2003).

Reyna and Farley (2006) suggest that evaluating a particular situation in an obsessive manner, may increase the risk of making errors on the part of the evaluator. In other words, if the individual spends too much time and energy evaluating the various possible costs and benefits when making a decision, he or she may increase the risk of making mistakes. Hence, intelligent risk taking may actually be a positive action, adapted by entrepreneurs as a skill. The skills which may be required by entrepreneurs include the ability to react well in highly unpredictable, uncertain, and rapidly changing environments (Picot et al. 2005). For an entrepreneur to be successful, it may be necessary to divert from certain well-learned or routine actions or protocols and show risk taking behavior (Baron 2004). For example, in a hypothetical job opportunity, an entrepreneur may be more successful if he or she uses intuitive skills more than simply analyzing the situation cognitively (Mitchell, et. al., 2007). Based on our review, there is no evidence from risk taking of entrepreneurs and similarly risk taking is considered as a negative cognitive function. In the present study, a simple neurocognitive task was used to measure risk taking behavior in entrepreneurs.

In a pilot study, we have recruited 20 entrepreneurs (17 male and 3 female, mean age = 24.04± 5.76 years). They were Directors of Business Incubators based at Shahid Beheshti University and the University of Tehran in December 2011. The Incubator system was adapted in Iran by Ministry of Science, Research and Technology (2001), based on the support system for entrepreneurs offered in Europe, United States and South and East Asia. The incubator is defined as: "an organization designed to accelerate the growth and success of entrepreneurial companies through an array of business support resources and services that could include physical space, capital, coaching, common services, and networking connections" (Jones Christensen, et. al., 2010). A group of 20 participants were matched for sex and age from

among postgraduate students in Shahid Beheshti and Tehran Universities.

The Balloon Analogue Risk Taking Test (BART) was used for evaluation of risk taking in the present experiment. This test was first used by Lejuez et al. (2003) in the University of Maryland to show "actual risk taking behavior in real circumstances".

In this task, participants engage in a computer simulation where a balloon is pumped in order to collect money. Each click on the pump inflates the balloon and makes it look bigger. With each pump, 50 tomans (monetary unit in Iran) is collected by the participant and the collected amount is shown on the screen. If the balloon explodes, all the money is lost, and the next uninflated balloon appears on the screen. At any point during the trial, the participant has the choice to stop pumping the balloon and click the collect money button. Clicking this button would transfer all the money to a permanent box. After each balloon explosion or money collection, the trial ends and a new trial begins with a new balloon appearing on the screen. A total of 30 balloons were inflated (Figure1).

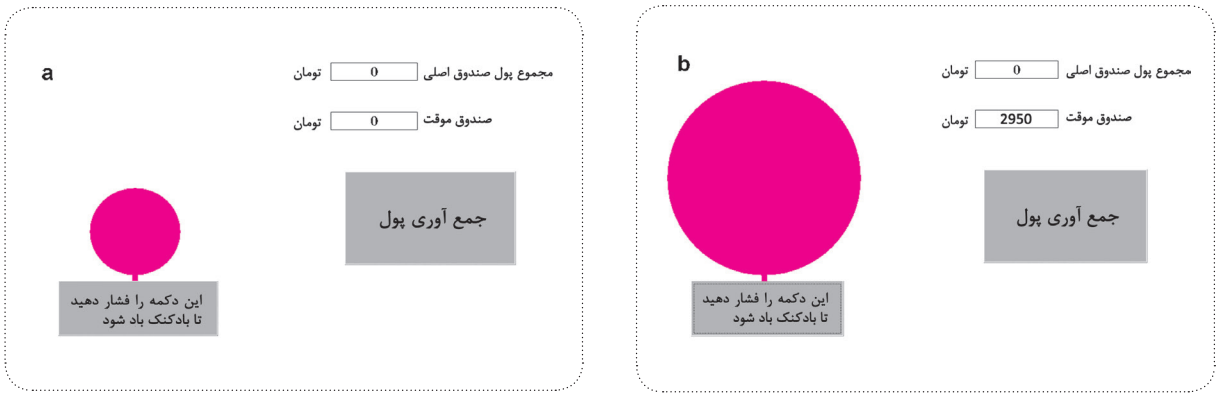

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Figure 1. Screen of BART task, a; starting position, b; risk taking position

Participants were told that at some point each balloon would explode and that this explosion could occur as early as the first pump all the way up to the point at which the balloon had expanded to fill the entire computer screen. The BART was designed to provide a context in which actual risky behavior could be examined (Vigil-Colet, 2007; Ravenzwaaij, Dutilh & Wagenmakers, 2011; Khodadadi, Dezfouli, Fakhari & Ekhtiari, 2010).

The result of various studies indicated that the BART may be a useful tool in the assessment of risk taking. In fact, there is a high correlation between Zukerman's sensation seeking test and risk taking test in real life situations (Lejuez et. al., 2003).

Results are shown in table 1. As can be seen, the number of pumps exerted by participants on the exploded balloons is significantly higher in the entrepreneurs group. On the other hand, the non-entrepreneurs showed a higher tendency for saving money. Number of pumps exerted on the whole balloon was marginally significant so that entrepreneurs have higher grade in this variable. Other dependent variables, including maximum and minimum number of pumps were not significantly different between the two groups.

To our best knowledge, present study is the first study that used BART in entrepreneurs. The results of the current study indicate that the number of pumps exerted on the exploded balloons was higher in the entrepreneur group and the number of attempts to save money was higher in the non-entrepreneurs group. Hence, it may be concluded that the level of risk taking tendency is higher in entrepreneurs. Furthermore, non-entrepreneurs inclined to save more money (i.e. take less risks) as compared to entrepreneurs who were likely to collect money by taking more risks.

It is interesting to note that some researchers have tried to explain entrepreneurial behavior by pointing out to certain personality correlates. For example, Zabel et al. (2008) argued that sensation seeking, defined as the tendency to experience new situations, may be a salient

Table 1. Comparing results of BART between entrepreneurs and non-entrepreneurs

Test Variables	Entrepreneurs Mean (SD)	Non Entrepreneurs Mean (SD)	T- Ratio	P- Value
Number of pumping on the exploded balloons	10.76 (5.83)	7.15 (3.28)	3.36	0.024
Number of pumping on the whole balloons	32.84 (11.42)	25.44 (11.09)	1.99	0.054
Number of decisions to save money	20.11 (5.01)	23.20 (2.87)	-2.33	0.025
Maximum number of pumps exerted	71.29 (29.87)	56.80 (22.83)	1.67	n.s.
Minimum number of pumps exerted	4.35 (4.03)	2.45 (3.36)	1.566	n.s.

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part of the personality profile of entrepreneurs. Sensation seeking has also been correlated with risky decision making.

Entrepreneurs some times have to make decisions under extreme uncertainty and ambiguity and this characteristic may partly explain entrepreneurial success (McVea, 2009). A new theory of entrepreneurship, states that entrepreneurs are less likely to avert risks and are inclined to face up to or indeed welcome various situations which may elicit risky behaviors (Newmann, 2007). This is in contrast to regular workers who may avert risky situations in favor of the status quo (e.g. assurance that wages are maintained). This theory is supported using an experimental paradigm in the present study.

Mullins and Forlani (2000) studied the possible risky situations in entrepreneurial ventures and found that entrepreneurs tend to choose various ventures and projects on the basis of the amount of risk involved in these ventures and projects rather than relying on purely logical situational and/or perceptual analyses. In other words, the greater risk potentials of a situation, the more likely that the person would select that particular situation.

Some researchers have suggested that intuition may play an important part in entrepreneurial thinking (Dijksterhuis, Bos, van der Leij, & van Baaren, 2009). It is argued that in rational thinking we may lose a huge amount of information which is available in the form of intuitive and subconscious (i.e. impressions and hunches which are gained through experience) thinking. An example is creative thinking during sleep when rational thinking is "switched off", and the unconscious information may have greater freedom and may be used for creative thinking (Chavez-Eakle & Sanchez, 2011). Future research on entrepreneurship should address the possible role played by intuitive thinking in decision making of entrepreneurs. Indeed, Khatri and Ng (2000) have already shown that intuition may play a role in strategic decision-making. Furthermore, Levander and Raccuia (2001) have shown that in entrepreneurial personality, rationality may have a lower priority than instinct in shaping entrepreneurs' behaviors.

In the current study, risk taking tendency was found to be higher in entrepreneurs than non-entrepreneurs and thus risk taking behavior may be a key factor in the screening and selection of entrepreneurs. One limitation of the present study is lack of an objective test for evaluation of entrepreneurship that should be considered in future studies along with a larger sample size.

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References

Baron, R. A. (2004). The cognitive perspective: a valuable tool for answering entrepreneurship's basic "why" questions. Journal of Business Venturing 19:221–239.

Blum, R., & Nelson-Mmari, K. (2004). The health of young people in a global context. Journal of Adolescent Health, 35: 402–418.

Caliendo, M., Fossen, F. M., & Kritikos, A. S. (2009). Risk attitudes of nascent entrepreneurs – New evidence from an experimentally-validated survey. Small Business Economics, 32(2), 153–167.

Chavez-Eakle, R. A., Sanchez, F. R. C. (2011). Beyond incubation: Creative breakthroughs associated with sleep . Sleep Medicine, 12(4):313-314.

Dijksterhuis, A., Bos, M. W., van der Leij, A., & van Baaren, R. B. (2009). Predicting soccer matches after unconscious and conscious thought as a function of expertise. Psychological Science, 20, 1381–1387.

Dworkin,J.(2005).Risktakingasdevelopmentallyappropriate-experimentationforcollegestudents. Journal of Adolescent Research, 20: 219–241.

Furnham, A., Nederstrom, M. (2010). Ability, demographic and personality predictors of creativity.Personality and Individual Differences 48: 957–961.

Glimcher, P. W., Camerer, C. F., Fehr, E., Poldrack, R. A. (2009). Introduction: a brief history of neuroeconomcis. In: Glimcher, P.W., Camerer, C.F., Fehr, E., Poldrack, R.A. (Eds.), Neuroeconomics. Decision Making and the Brain. Elsevier, London.

Jones Christensen, L., Parsons, H. and Fairbourne, J. (2010) Building entrepreneurship in subsistence markets: Microfranchising as an employment incubator. Journal of Business Research, 63, 595-601.

Khodadadi A, Dezfouli A, Fakhari P, Ekhtiari H. (2010) Effects of Methadone Maintenance Treatment on Decision-Making Processes in Heroin-Abusers: A Cognitive Modeling Analysis. Behavioural an Clinical Neuroscience, 1 (3), 44-49

Knutson, B., Bossaerts, P. (2007). Neural antecedents of financial decisions. Journal of Neuroscience 27: 8174–8177.

Krueger, N. (2007). What lies beneath: The experiential essence of entrepreneurial thinking, Entrepreneurship Theory & Practice, 31(1), 123-138.

Lejuez, C. W., Aklin, W. M., Zvolensky, M. J., Pedulla, C. M. (2003). Evaluation of the Balloon Analogue Risk Task (BART) as a predictor of adolescent real-world risk-taking behaviours. Journal of Adolescence, 26(4): 475-479.

McVea, J. F. (2009). A field study of entrepreneurial decision-making and moral imagination. Journal of Business Venturing 24: 491–504.

Michalski, R. L., Shackelford, T. K. (2010). Evolutionary personality psychology: Reconciling human nature and individual differences. Personality and Individual Differences, 48(5): 509-516.

Mitchell, R. K., Busenitz, L., Bird, B., Gaglio, C., McMullen, J., & Morse, E. (2007). The central question in entrepreneurial cognition research, Entrepreneurship Theory & Practice, 31(1), 1-27.

Newman, A. F. (2007). Risk-bearing and entrepreneurship. Journal of Economic Theory 137: 11 – 26.

Picot, A., Reichwald, R., Wigand, R. T. (2008). Information, Organization and Management. Springer, Berlin.

Plassmann, H., O'Doherty, J., Rangel, A. (2007). Orbitofrontal cortex encodes willingness to pay in everyday economic transactions. Journal of Neuroscience 27: 9984–9988.

Platt, M.L., Huettel, S.A. (2008). Risky business: the neuroeconomics of decision making under uncertainty. Nature Neuroscience 11: 398–403.

Prabhakaran, R., Kraemer, D. J. M., & Sharon L. (2011). Approach, avoidance, and inhibition: Personality traits predict cognitive control abilities. Personality and Individual Differences, Corrected Proof.

Rangel, A., Camerer, C., Montague, P. R. (2008). A framework for studying the neurobiology of value-based decision making. Nature Review Neuroscience 9: 545–556.

Ravenzwaaij, D. V., Dutilh, G., & Wagenmakers, E. (2011). Cognitive model decomposition of the BART: Assessment and application Original Research Article Journal of Mathematical Psychology, 94-105

Schultz, W. (2006). Behavioral theories and the neurophysiology of reward. Annual Review Psycholology 57: 87–115.

Vigil-Colet, A. (2007). Impulsivity and decision making in the balloon analogue risk-taking task. Personality and Individual Differences, 43(1): 37-45.

White, R. E., Thornhill, S., & Hampson, E. (2006). Entrepreneurs and evolutionary biology: The relationship between testosterone and new venture creation. Organizational Behavior and Human Decision Processes, 100: 21–34.

White, R. E., Thornhill, S., & Hampson, E. (2007). A biosocial model of entrepreneurship: The combined effects of nurture and nature. Journal of Organizational Behavior, 28: 451–466.

Williams, P., Holmbeck, G., & Greenley, R. (2002). Adolescent health psychology. Journal of Consulting and Clinical Psychology, 70: 828–842.

Zabel, K. L., Christopher, A. N., Marek, P., Wieth, M. B., Carlson, J. J. (2009). Mediational effects of sensation seeking on the age and financial risk-taking relationship. Personality and Individual Differences, 47(8): 917-921.

Zhao, H., & Seibert, S. E. (2006). The big five personality dimensions and entrepreneurial status: A meta-analytical review. Journal of Applied Psychology, 91: 259–271.

Evaluation of the Functional Recovery in Sciatic Nerve Injury following the Co-transplantation of Schwann and Bone Marrow Stromal Stem Cells in Rat

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ABSTRACT

Introduction: Transplantation of bone marrow stromal cells (BMSCs) or Schwann cells (SCs) can increase axonal regeneration in peripheral nerve injuries. Based on our previous investigations, the goal of the present work was to examine the individual and synergistic effects of the two different cell types in sciatic nerve injury . We pursued to evaluate the effects of BMSCs and SCs co-transplantation on the functional recovery after sciatic nerve injury in rat.

Methods: In this experimental research, adult male Wistar rats (n=32, 250-300g) were used, BMSCs and SCs were cultured, and the SCs were confirmed with anti S100 antibody. Rats were randomly divided into 4 groups (n=8 in each group): 1- control group: silicon tube filled with fibrin gel without cells; 2- BMSCs group: silicon tube filled with fibrin gel seeded with BMSCs; 3- SCs group: silicon tube filled with fibrin gel seeded with SCs and 4- co-transplantation group: silicone tube filled with fibrin gel seeded with BMSCs and SCs. The left sciatic nerve was exposed, a 10 mm segment removed, and a silicone tube interposed into this nerve gap. BMSCs and SCs were transplanted separately or in combination into the gap. BMSCs were labeled with anti-BrdU and SCs were labeled with DiI. After 12 weeks electromyographic and functional assessments were performed and analyzed by one-way analysis of variance (ANOVA).

Results: Electromyographic and functional assessments showed a significant difference between the experimental groups and controls. Electromyography measures were significantly more favourable in SCs transplantation group as compared to BMSCs transplantation and co-transplantation groups ($p<0.05$). Functional assessments showed no statistically significant difference among the BMSCs, SCs and co-transplantation groups ($p<0.05$).

Discussion: Transplantation of BMSCs and SCs separately or in combination have the potential to generate functional recovery after sciatic nerve injury in rat. The electromyography evaluation showed a greater improvement after SCs transplantation than BMSCs or the co-transplantation of BMSCs and SCs.

Key Words:
Bone Marrow Stromal Cells,
Schwann Cells,
Transplantation,
Peripheral Nerve Regeneration.

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1. Introduction

Peripheral nerve system (PNS) has the potential to regenerate nerve cells, and the peripheral nerve injury has been successfully recovered using various procedures such as nerve autograft or nerve guidance tubes (Belkas, Shoichet, & Midha, 2004). In peripheral nerve injury, one of the problems is suturing nerve ends when the resulting gap is too long (Millesi, 1984). The nerve ends can be connected with a nerve autograft to provide a guidance for the regenerating nerves. However, for more extensive nerve trauma, a longer graft is needed, and when the graft is thinner than the injured nerve, the transplantation of a bundle of nerve fibers becomes mandatory. Since the procedure requires a large graft from a healthy nerve, sensory and motor destruction may occur at the donor site (Ide, 1996; Ishikawa et al., 2009).

Axonal regeneration in a peripheral nerve injury which requires extrinsic factors to promote growth and supply guidance to the target. To overcome these problems, a variety of nerve guide tubes have been used to facilitate cell transplantation. The purposes of the cellular transplantation include: 1- bridging the gap; 2- providing a suitable environment to induce axonal regeneration and 3- to promote neovascularization. Different procedures have been used to improve regeneration of peripheral nerves. One of those is the seeding of the cells into the guide tubes (Belkas et al., 2004; Dezawa, 2005; Fan, Crawford, & Xiao, 2011; Ishikawa et al., 2009).

Bone marrow stromal cells (BMSCs) and Schwann cells (SCs) are cells with the capability to produce nerve growth factors such as nerve growth factor (NGF), brain-derived nerve growth factor (BDNF) and vascular endothelial growth factor (VEGF) . These factors play an important role in the survival and proliferation of axons. Thus BMSCs and SCs transplantation may possibly result in the recovery of peripheral nerves following injury (Braga-Silva et al., 2006; C. J. Chen et al., 2007; Lu et al., 2006; Schlosshauer, Muller, Schroder, Planck, & Muller, 2003).

Our previous study showed that BMSCs and SCs can be effective on functional recovery of the sciatic nerve injury on their own (Zarbakhsh et al., 2012). To consolidate the earlier findings, here we pursued to compare the effects of the co-transplantation of these cells (BMSCs and SCs) with sole transplantation of these cells on the peripheral nerve recovery as this has not so far been evaluated under similarly controlled conditions.

2. Methods

In this experimental research, male Wistar rats (n=32, 250-300g) bred in Tehran Pasteur Institute were used. All animals had free access to laboratory chow, and tap water. Rats were randomly divided into 4 groups (n=8 in each group): 1- control group; 2- BMSCs transplantation group; 3- SCs transplantation group and 4- Co-transplantation group. All procedures in this study, including the use and care of animals, were approved by the Research Council of Tehran University of Medical Sciences (Tehran, Iran).

2.1. BMSCs Culture

Briefly, to obtain BMSCs, rats were killed and femurs and tibias were dissected out. The marrow was extruded with 10 ml of Dulbecco`s Modified Eagle Medium (DMEM) (Sigma, Aldrich) and cultured in DMEM (Azizi, Stokes, Augelli, Digirolamo, & Prockop, 1998). BMSCs were subcultured four times and were labeled with anti-BrdU antibody (Bromodeoxyuridin) (Sigma Aldrich) as the primary antibody and rhodamine (Sigma Aldrich) as the secondary antibody in the sciatic nerve. (Fig. 1, 2) (Li et al., 2006; Liao et al., 2001; Zurita & Vaquero, 2006; Zarbakhsh et al., 2012).

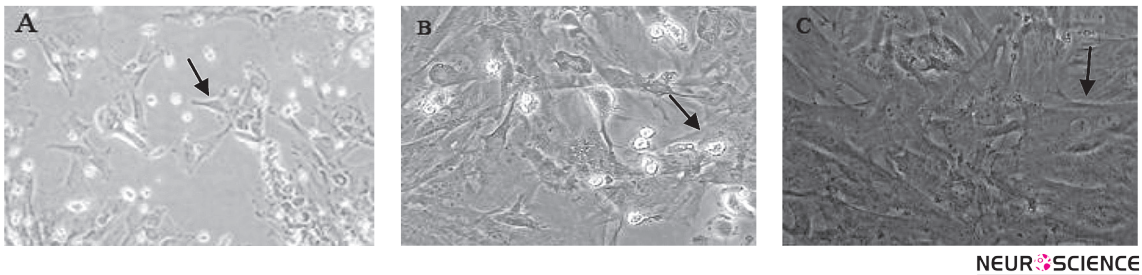

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Figure 1. Cultured bone marrow stromal cells. (A) In P0 stage adherent cells exhibited small round, spindle-shaped (arrow) (×200). (B) In P2 stage. Most cells grew, and exhibited fibroblast-like morphology. The small round cells adhered to the surface of these cell layers (arrow) (×200). (C) In P4 stage round cells disappeared and the fibroblast-like cells became morphologically homogeneous (arrow) (×200).

2.2. SCs Culture

Briefly, to obtain SCs, rats were killed and their sciatic nerves were dissected bilaterally. After removing epineurium and connective tissue, the sciatic nerves were cut into 2-3 mm fragments and cultured in DMEM (Zurita, Bonilla, Otero, Aguayo, & Vaquero, 2008). SCs were subcultured three times and were confirmed by anti S100 antibody. Dilution range of anti S100 antibody was ratio of 1 to 500 of rabbit anti S100 antibody (Sigma) in (PBS+0.3% Triton X+10% Normal Goat serum) (Fig. 3) (Rodriguez, Verdu, Ceballos, & Navarro, 2000). SCs were labeled with DiI (1,1`-dioctadecyl-3,3,3`,3`-tetramethylindocarbocyanine perchlorate) (Sigma Aldrich) (170 mg/ml in DMSO and diluted 1:10 in saline) in the sciatic nerve. Briefly SCs were suspended in DMEM and 5µl/ml DiI was added. After incubation for 20 min, the cells were centrifuged and washed twice with PBS. Then they were resuspended in PBS for transplantation. 4 weeks after the transplantation, prepared frozen sections and the labeled cells were detected using the fluorescent microscope (Olympus AX70) (Fig. 4) (Li et al., 2008; Pourheydar et al., 2012; Al et al., 2007; Bakhtyari et al., 2009; Haastert et al., 2006, Zarbakhsh et al., 2012).

2.3. Transplantation Procedure

Rats were anesthetized and after skin incision, the sciatic nerve was exposed using a muscle splitting incision. Under an operating microscope the left sciatic nerve was exposed at the mid-thigh, and a 10 mm segment of the nerve was removed. A 12 mm silicone tube (1 mm inner diameter, 2 mm outer diameter) was interposed into this nerve gap (Y. S. Chen et al., 2000). Both proximal and distal ends of the nerve were anchored into the conduit with 10-0 nylon suture (Fig.5). The silicone tube in the BMSCs group was filled with fibrin gel seeded with about 500,000 BMSCs, in the SCs group was filled with fibrin gel seeded with about 500,000 SCs, in the co-transplantation group was filled with fibrin gel seeded with about 250,000 BMSCs and 250,000 SCs; and the control group with fibrin gel without any cell. Finally the skin was sutured with 5-0 silk.

2.4. Electromyography (EMG) Study

Twelve weeks after the transplantation, rats were anesthetized and the sciatic nerves were exposed. Electric stimulation was utilized to the proximal site of the injured nerve. The compound muscle action potential was recorded in the gastrocnemius with a needle electrode and a reference cap electrode inserted at the knee joint. The stainless steel needle used as the ground electrode was inserted into the tail skin (Chen et al., 2007; Mimura, Dezawa, Kanno, Sawada, & Yamamoto, 2004; Zarbakhsh et al., 2012).

2.5. Functional Assessment

The functional assessment of the sciatic nerve regeneration was expressed by the sciatic function index (SFI). Briefly, twelve weeks after the transplantation, rats hind feet were dipped in ink and the rats were allowed to walk through a plastic tunnel so that the footprints could be recorded on paper loaded onto the bottom of the tunnel. The distance among the fingers, toes

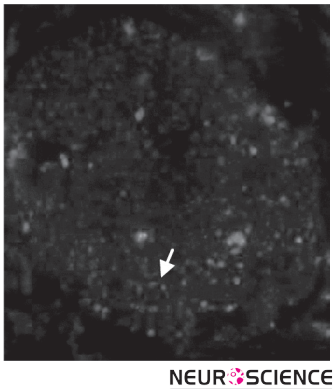

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Figure 2. Cross-section of the sciatic nerve, 4 weeks after the transplantation of BMSCs labeled with anti BrdU antibody shown as red spots in the sciatic nerve (arrow) (×100).

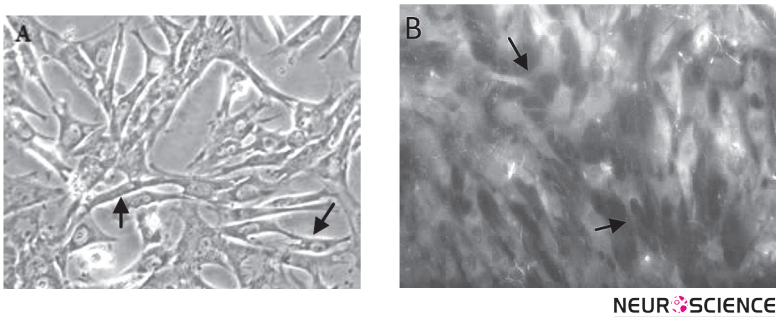

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Figure 3. (A) Cultured Schwann cells were small, elongated, and spindle shaped in P2 stage (arrows) (×200). (B) Schwann cells were labeled with anti S100 antibody, and showed a tendency to line up side by side or end to end and to form interconnected networks (arrows) (×200).

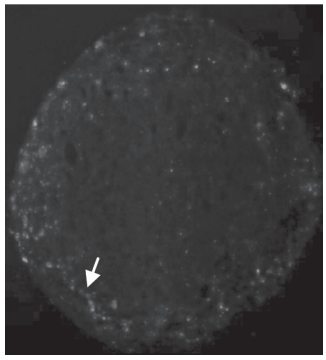

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Figure 4. Cross-sections of the sciatic nerve 4 weeks after the transplantation of Schwann cells labeled with DiI shown as red spots in the sciatic nerve (arrow) (×100).

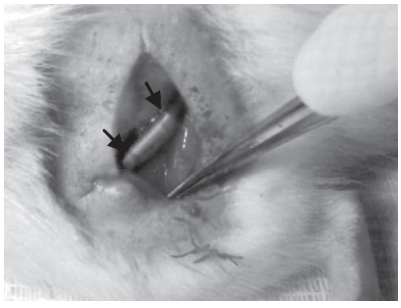

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Figure 5. Nerve graft. The left sciatic nerve was exposed at the mid-thigh and a 10 mm segment of the nerve was removed. A 12 mm silicone tube was interposed into this nerve gap. Arrows show two heads of the nerve in the silicone tube.

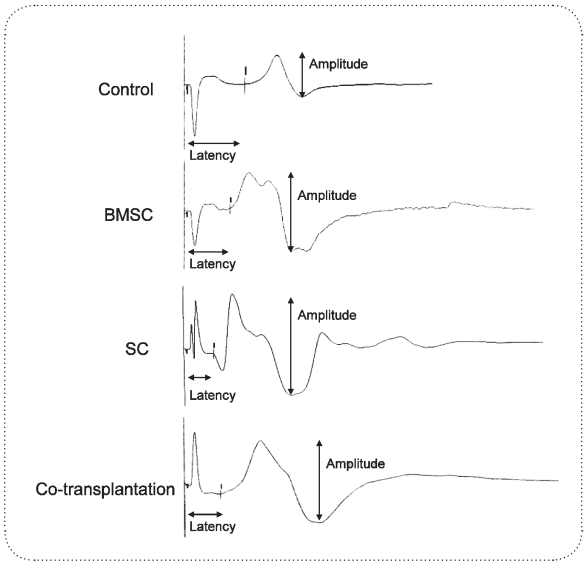

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Figure 6. Electromyographic waves for control, BMSCs, SCs and co-transplantation groups 12 weeks after surgery. Amplitude and latency are shown for each group. The time calibration bar was 2 ms and the amplitude calibration bar was 10 mV. The stimulation intensity was 2.3 mA and the duration was 0.1 ms.

and heels was measured. The SFI was calculated as follow: SFI= -38.3×(EPL-NPL)/NPL+109.5×(ETS-NTS)/NTS+13.3×(EITS-NITS)/NITS-8.8. In general, the SFI oscillates around 0 for normal nerve function, whereas around -100, the SFI indicates a total dysfunction (Chen et al., 2007; Mimura et al., 2004; Zarbakhsh et al., 2012).

2.6. Statistical Analyses

All data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey test. Obtained data were presented as means ± standard error and a level of $p<0.05$ was considered statistically significant.

3. Results

3.1. BMSCs Culture

BMSCs obtained from the femurs and tibias of adult rats comprised heterogeneous groups of cells after seeding and growing in culture plates. After initial plating, the adherent cells exhibited a small rounded-shape, a spindle-shape or a large flattened morphology (Fig.1A). Most cells grew and exhibited a fibroblast-like morphology on reaching confluence. The small rounded cells adhered to the surface of these cell layers (Fig.1B). These rounded cells disappeared after repeated passages, whereas the fibroblast-like cells became enriched. Upon the 4th passage, the fibroblast-like cells became morphologically homogeneous (Fig.1C).

3.2. Labeled BMSCs in the Sciatic Nerve Tissue

Immunohistochemistry technique showed the BMSCs labeled with the anti-BrdU antibody were red spots in the cross section of the sciatic nerve (Fig.2). Presence of the red color was due to the use of rhodamine as the secondary antibody. Our results confirmed not only the the presence but the viability of the transplanted cells in the silicone tube bridging the gap 4 weeks after the transplantation.

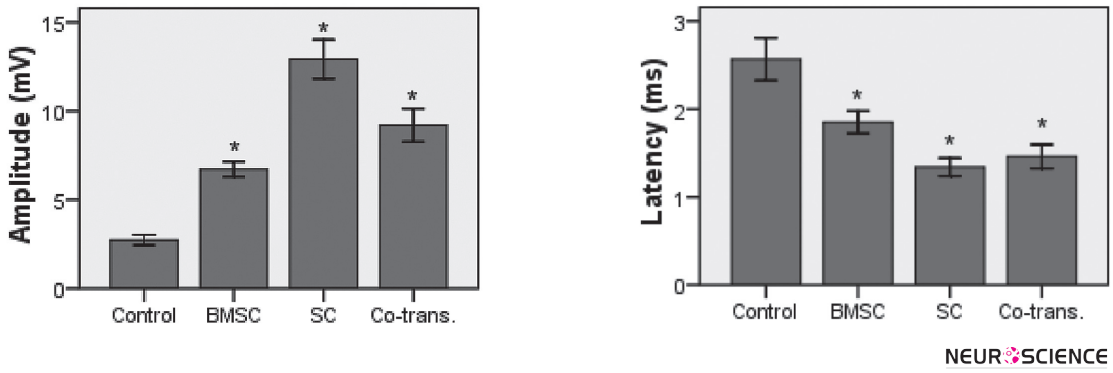

Figure 7. The results of electromyography tests of amplitude and latency showed there were statistically significant differences between the control and experimental groups (BMSCs, SCs and co-transplantation of BMSCs and SCs)* and SCs transplantationresulted in a more favorable results compared to the other groups (*p<0.05).

3.3. SCs Cultured

The spindle-shaped cellular morphology of the SCs seen on the culture plate was viable. Most of the cells were small, elongated, and spindle shaped (Fig.3A). Fluorescence microscopy showed the SCs were S100-positive cells. In the culture dishes, the SCs had a tendency to line up side by side or end to end and to form interconnected networks (Fig.3B).

3.4. Labeled SCs in the Sciatic Nerve Tissue

Histochemistry technique showed the SCs labeled with DiI as red-positive spots in the cross section of sciatic nerve (Fig.4). The results confirmed the presence and viability of the transplanted cells in the silicone tube bridging the gap 4 weeks after the transplantation.

3.5. Electromyography (EMG)

The results of the EMG tests comprised both amplitude and latency measures. The time calibration bar was 2 milliseconds (ms) and the amplitude calibration bar was 10 millivolts (mV). The stimulation intensity was 2.3 milliampere (mA) and the duration was 0.1 ms (Fig.6).

The results showed a statistically significant difference between control and experimental groups (BMSCs, SCs and co-transplantation). Moreover, results from the SCs-transplant group were significantly more favorable compared to the other groups (p<0.05) (Fig.7).

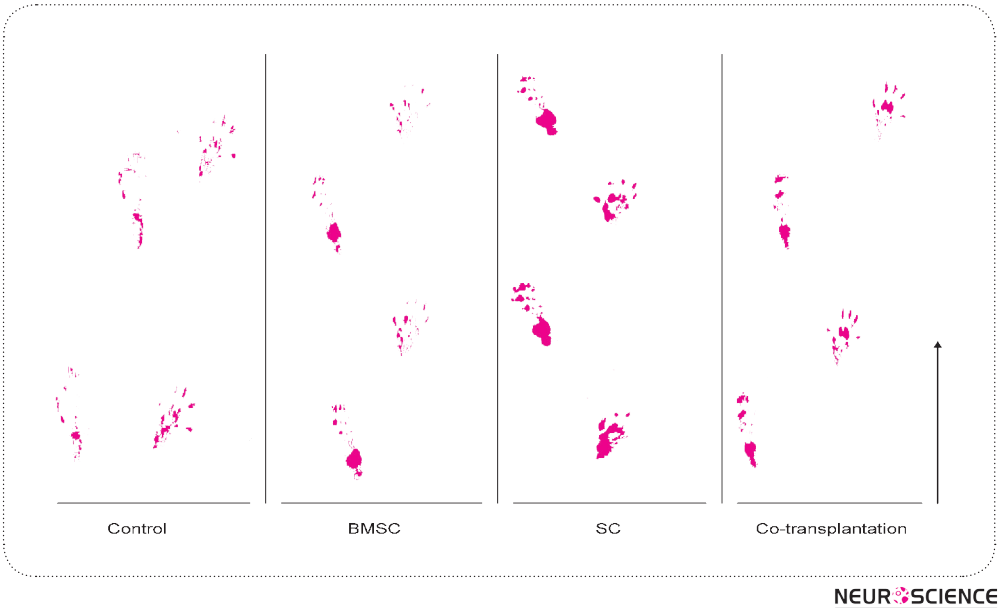

Figure 8. Foot print of control, BMSCs, SCs and co-transplantation groups 12 weeks after surgery. Arrow shows walking direction.

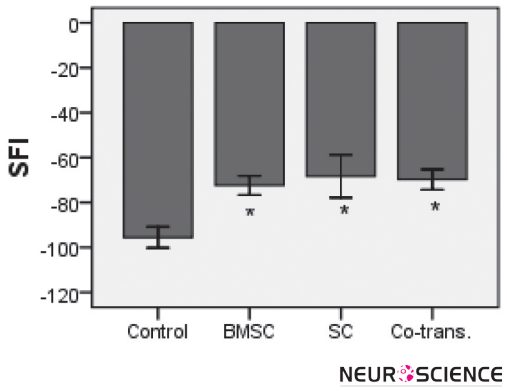

Figure 9. The results of the sciatic function index (SFI) test showed there were statistically significant differences between control and experimental groups (BMSCs, SCs and co-transplantation of BMSCs and SCs)* while showing no statistically significant difference among the experimental groups (*p<0.05).

3.6. Functional Analysis

The results of SFI showed a statistically significant difference between the control and experimental groups (BMSCs, SCs and co-transplantation), however, no statistically significant difference was observed amongst the experimental groups (p<0.05) (Fig.8, 9).

4. Discussion

Peripheral nerve injury is considered as one of the most challenging microsurgical problems. These damages may lead to a considerable disability due to the loss of both motor and sensory functions. Since the auto graft procedure involves multiple surgeries, loss of function, and loss of sensation at the donor site, development of a high quality replacement for auto grafts is required (Belkas et al., 2004; Dezawa, 2005; Ishikawa et al., 2009).

In the previous study we compared the effects of transplantation of BMSCs and SCs on the recovery of rats' sciatic nerve injury. We also showed that BMSCs and SCs can be effective by their own and SCs were significantly more effective than the BMSCs (Zarbakhsh et al., 2012). In this study, we compared the effects of the sole transplantation of BMSCs, SCs and the co-transplantation of these cells on the recovery of rats' sciatic nerve injury under similar condition. Several investigators have shown that BMSCs and SCs can repair the sciatic nerve injuries (Braga-Silva et al., 2006; C. J. Chen et al., 2007; Lu et al., 2006; Schlosshauer et al., 2003), however, BMSCs and SCs have not been used in combination for the same purpose. Comparing the effects these cells' co-transplantation (BMSCs and SCs) with the effect of their sole transplantation on the recovery of the injured peripheral nerve under similar conditions may introduce a novel clinical approache in utilizing these cells to replace peripheral nerve recovery auto grafts.

Due to the benefits of stem cells and Schwann cells in producing nerve growth factors such as NGF, BDNF and VEGF, as well as substantiating extracellular matrix proteins such as collagen IV and laminin (Braga-Silva et al., 2006; C. J. Chen et al., 2007; Fan et al., 2011; Feng, Zhou, Rush, & Ferguson, 2008; Ide, 1996), there is good evidence to support the hypothesis that transplantation of BMSCs and SCs may repair peripheral nerve injuries. Moreover, using the co-transplant of these cells may possibly be an important step in the selection of a repair procedure based on the peripheral nerve recovery measures.

In this study, we showed that the sole- and co-transplantation of BMSCs and SCs may lead to functional recovery of the injured sciatic nerve. This was documented by the EMG test in the gastrocnemius muscle as well as the walking behavior measured by the foot print analysis. The results of the EMG tests revealed that there were statistically significant differences between the control and experimental groups (BMSCs, SCs and co-transplantation of these cells). Some other reported data support our findings (C. J. Chen et al., 2007; Murakami et al., 2003; Rodriguez et al., 2000; Wang et al., 2008). The greater recovery in the SCs group as compared to the BMSCs group could probably be due to the direct and essential role of SCs in regeneration and recreation of axonal bridges. Similarly, The greater recovery in the SCs group as compared to the co-transplantation group was probably due to the number of SCs in SCs group than the co-transplant group. Furthermore, in the co-transplant group these cells might have overlapped resulting in a less repaire rate. The results of the SFI tests

showed there were statistically significant differences between the control and experimental groups. These results are in agreement with findings of other investigations (C. J. Chen et al., 2007; Hou, Zhang, Quan, Liu, & Zhu, 2006; Kim, Lee, & Lee, 2007; Nie et al., 2007), while the lack of any statistically significant difference amongst the experimental groups is a new finding and has not been previously addressed under similar conditions. Presumably after 12 weeks, due to the growing axons, a significance difference was observed in the results of EMG amongst the experimental groups, while myelin formation was not completed. Accordingly, there was no statistically significant difference amongst the experimental groups with regard to the SFI results. Nevertheless, we might possibly have observed a significance difference in the results of SFI amongst the experimental groups if waited longer.

Our results suggested that the sole- or co-transplantation of BMSCs and SCs have the potential to generate functional recovery of rats' injured sciatic nerve. EMG evaluation revealed that the SCs transplantation results in a greater functional improvement in the injured nerve as compared to BMSCs transplantation or co-transplantation of BMSCs and SCs. Our findings support use of SCs transplant as opposed to BMSCs or combination of BMSCs and SCs transplant for the clinical repair of the injured peripheral nerves.

References

Al, J., Epstein, P. N., Gozal, D., Yang, B., Wurster, R., & Cheng, Z., J. (2007). Morphology and topography of nucleus ambiguus projections to cardiac ganglia in rats and mice. Neuroscience, 149, 845-860.

Azizi, S. A., Stokes, D., Augelli, B. J., Digirolamo, C., & Prockop, D. J. (1998). Engraftment, and migration of human bone marrow stromal cells implanted in the brains of albino rats similarities to astrocytes grafts. Proc Natl Acad Sci USA, 95(7), 3908-3913.

Bakhtyari, M., Abootaleb, H., & Mansouri, K. (2009). Electrophysiological study of sciatic nerve regeneration through tubes seeded with schwann cells. Iranian Journal of Neuroscience, 1(3), 49-56.

Belkas, J. S., Shoichet, M. S., & Midha, R. (2004). Peripheral nerve regeneration through tubes. Neural Res, 26(2), 151-160.

Braga-Silva, J., Gehlen, D., Roman, J. A., Menta, C., Atkinson, E. A., Machado, D. C., et al. (2006). Bone marrow stem cells and platelet-rich plasma effects on nervous regeneration and functional recovery in an acute defect model of rats` peripheral nerve. Acta Ortop Bras, 14(5), 273-275.

Chen, C. J., Ou, Y. C., Liao, S. L., Chen, W. Y., Chen, S. Y., Wu, C. W., et al. (2007). Transplantation of bone marrow stromal cells for peripheral nerve repair. Exp. Neurology, 204(1), 443-453.

Chen, Y. S., Hsieh, C. L., Tasi, C. C., Chen, T. H., Cheng, W. C., Hu, C. L., et al. (2000). Peripheral nerve regeneration using silicone rubber chambers filled with collagen, laminin and fibronectin. Biomaterials, 21(15), 1541-1547.

Dezawa, M. (2005). Future views and challengges to the peripheral nerve regeneration by cell based therapy. Rinsho Shinkeigaku (Japanes), 45(11), 877-879.

Fan, W., Crawford, R., & Xiao, Y. (2011). The ratio of VEGF/PEDF expression in bone marrow mesenchymal stem cells regulates neovascularization. Differentiation, 81(3), 181-191.

Feng, S. Q., Zhou, X. F., Rush, R. A., & Ferguson, I. A. (2008). Graft of pre-injured sural nerve promotes regeneration of corticospinal tract, and functional recovery in rats with chronic spinal cord injury. Brain Res, 1209, 40-48.

Haastert, K., Lipokatic, E., Fischer, M., Timmer, M., & Grothe, C. (2006). Differentially promoted peripheral nerve regeneration by grafted Schwann cells over-expressing different FGF-2 isoforms. Neurobiology of Disease, 21(1), 138-153.

Hou, S. Y., Zhang, H. Y., Quan, D. P., Liu, X. L., & Zhu, J. K. (2006). Tissue engineered peripheral nerve grafting by differentiated bone marrow stromal cells. Neuroscience, 140(1), 101-110.

Ide, C. (1996). Peripheral nerve regeneration. Neurosci. Res., 25(2), 101-121.

Ishikawa, N., Suzuki, Y., Dezawa, M., Kataoka, K., Ohta, M., Cho, H., et al. (2009). Peripheral nerve regeneration by transplantation of BMSC-derived schwann cells as chitosan gel sponge scaffolds. Willey Periodicals, Inc. J Biomed Mater Res., 89(4), 1118-1124.

Kim, S. M., Lee, S. K., & Lee, J. H. (2007). Peripheral nerve regeneration using a three dimensionally cultured Schwann cell conduit. The Journal of Craniofacial Surgery, 18(3), 475-488.

Li, P., Akimoto, T., Zhang, M., Williams, R. S., & Yan, Z. (2006). Resident stem cells are not required for exercise-induced fiber-type switching and angiogenesis but are necessary for activity-dependent muscle growth. Am J Physiol Cell Physiol, 290(6), 1461-8.

Li, N., Yang, H., Lu, L., Duan, C., Zhao, C., & Zhao, H. (2008). Comparison of the labeling efficiency of BrdU, DiI and FISH labeling techniques in bone marrow stromal cells. Brain Research, 1215, 11-19.

Liao, G., Wu, F. Y., & Hayward, S. D. (2001). Interaction with the Epstein-Barr virus helicase target Zta to DNA replication compartments. J Virol, 75(18), 8792-802.

Lu, M. C., Chang, Y. H., Chiang, L. C., Wang, H. T., Cheng, C. Y., Yao, C. H., et al. (2006). Peripheral nerve regeneration through nerve guides filled with bilobalide and schwann cells. Biomed Eng Appl Basis Comn, 18(1), 8-12.

Millesi, H. (1984). Nerve grafting. Clin Plast Surg 11(1), 105-113.

Mimura, T., Dezawa, M., Kanno, H., Sawada, H., & Yamamoto, I. (2004). Peripheral nerve regeneration by transplantation of bone marrow stromal cell-derived Schwann cells in adult rats. J. Neurosurg, 101(5), 806-812.

Murakami, T., Fujimoto, Y., Yasunaga, Y., Ishida, O., Tanaka, N., Ikuta, Y., et al. (2003). Transplanted neuronal progenitor cells in a peripheral nerve gap promote nerve repair. Brain Res, 974(1-2), 17-24.

Nie, X., Zhang, Y. J., Tian, W. D., Jiang, M., Dong, R., Chen, J. W., et al. (2007). Improvement of peripheral nerve regeneration by a tissue-engineered nerve filled with ectomesenchymal stem cells. Int. J. Oral Maxillofac. Surg., 36(1), 32-38.

Pourheydar, B., Joghataei, M. T., Bakhtiari, M., Mehdizadeh, M., Yekta, Z., & Najafzadeh, N. (2012). Co-transplantation of bone marrow stromal cells with schwann cells evokes mechanical allodynia in the contusion model of spinal cord injury in rat. Cell Journal (yakhteh), 13(4), 213-222.

Rodriguez, F. J., Verdu, E., Ceballos, D., & Navarro, X. (2000). Nerve guides seeded with autologous Schwann cells improve nerve regeneration. Exp. Neurology, 161(2), 571-584.

Schlosshauer, B., Muller, E., Schroder, B., Planck, H., & Muller, H. (2003). Rat schwann cells in bioresorbable nerve guides to promote, and accelerate axonal regeneration. Brain Res, 963(1-2), 321-326.

Wang, D., Liu, X. L., Zhu, J. K., Jiang, L., Hu, J., Zhang, Y. J., et al. (2008). Bridging small-gap peripheral nerve defects using acellular nerve allograft implanted with autologous bone marrow stromal cells in primates. Brain Res, 1188, 44-53.

Zarbakhsh, S., Bakhtiari, M., Faghihi, A., Joghataie, M. T., Mehdizadeh, M., Khoei, S., et al. (2012). The effects of Schwann and bone marrow stromal stem cells on sciatic nerve injury in rat: A comparison of functional recovery. Cell Journal(Yakhteh) 14(1), 39-46.

Zurita, M., Bonilla, C., Otero, L., Aguayo, C., & Vaquero, J. (2008). Neural transdifferentiation of bone marrow stromal cells obtained by chemical agents is a short-time reversible phenomenon. Neuroscience Research, 60(3), 275-280.

Zurita, M., & Vaquero, J. (2006). Bone marrow stromal cells can achieve cure of chronic paraplegic rats: functional and morphological outcome one year after transplantation. Neurosci. Lett, 402(1-2), 51-56.

Antiepileptic and Antioxidant Effect of Hydroalcoholic Extract of Ferula Assa Foetida Gum on Pentylentetrazole-induced Kindling in Male Mice

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ABSTRACT

Introduction: Considering the prevalence of epilepsy and the failure of available treatments for many epileptic patients, finding more effective drugs in the treatment of epilepsy seems necessary. Oxidative stress has a special role in the pathogenesis of epileptic syndrome. Therefore, in the present study, we have examined the anti-epileptic and anti-oxidant properties of the Ferula Assa Foetida gum extract, using the pentylentetrazole (PTZ) kindling method.

In this experimental study, sixty male Albino mice weighing 25-30 g were selected and were randomly divided into 6 groups. 1- the control group, 2- PTZ-kindled mice, 3- positive control group which received valproate (100 mg/kg) as anti-convulsant drug, 4-5 & 6- the groups of kindled mice that pretreated with 25, 50 and 100 mg/kg doses of Ferula Assa Foetida gum extract.

Methods: Kindling has been induced in all groups, except for the control group via 11 PTZ injections (35 mg /kg; ip) every other day for 22 days. In the 24th day, the PTZ challenge dose was injected (75 mg / kg) to all groups except the control group. The intensity of seizures were observed and noted until 30 minutes after PTZ injection. At list, the mice were decapitated and the brains of all the mice were removed.. and their biochemical factors levels including malondialdehyde (MDA), superoxide dismutase (SOD) and nitric oxide (NO) were determined.

Results: Results of this study show that Ferula Assa Foetida gum extract is able to reduce seizure duration and its intensity. In addition, this extract has reduced MDA and NO levels and increased the level of SOD in the brain tissue compared to the PTZ- kindled mice.

Discussion: It can be concluded that Ferula Assa Foetida gum extract, in specific doses, is able to show an anti-epileptic effect because of its antioxidant properties, probably acting through an enzyme activity mechanism.

1. Introduction

Epilepsy is one of the most serious neurological disorders, which is induced by a sudden increment of stimulatory factors in the cortical neurons. About 0.5-3% of people experience it during their lifetime (Theodore & Fisher, 2007; Levav, Stephenson & Theodore, 1999). Epilepsy refers to a functional disorder of the brain with the occurrence of recurrent and unpredictable seizures (McNamara, 1994). Among the reasons of seizure attack are brain injury, stroke, serious events before birth, brain tumor, infection (encephalitis, bacterial meningitis) and genetic factors (Khaleghi-Ghadiri & Gorji, 2004).

It has been known that during epileptic attack, oxidative stress occurs, free radicals are produced and membrane lipid peroxidation happens, all of which cause

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tissue damage (Ilhan et al., 2005; Tuszkiewicz-Misztal, Opoka-Winiarska &, Postêpski, 2000).

MDA is the end product of unsaturated membrane fatty acid peroxidation and therefore, has been considered as the sign of lipid peroxidation (Chauhan, & Chauhan, 2006). NO is also one of the most abundant free radicals in the body (Evereklioglu et al., 2004) which its rapid reaction with other free radicals such as superoxide anion induces oxidizing product, neuronal toxicity and epileptic attack (Sogut et al., 2003; Buisson et al., 1993). In the presence of superoxide free radicals, the activity of SOD increases (Rukmini, Souza & Souza, 2004). Superoxide radical is also one of the free radicals which indirectly trigger lipid peroxidation (Dehpour et al., 2009). SOD can scavenger the superoxide anion by catalyzing it to H2O2 and O2 by which prevent the oxidative stress-induced cellular damage (Sogut et al., 2003). Despite the development of various anti-epileptic drugs, about one third of epileptic patients are resistant to current drug therapies. The resulted side effects from common toxic anti-epileptic drugs also lead to limited use of these drugs and failure of their optimal therapeutic effect in the treatment. Thus, considering the mentioned reports, focusing on new anti-epileptic drugs is necessary (Zargari, 1991).

In the recent years, there has been a revival in the use of medicinal plants. The milder side effects of these plants, the variety of effective compounds in plants, the recommendations of the World Health Organization for using medicinal plants have all contributed to the growth of this new trend (Zamani, 1999). In the Iranian and Indian traditional medicine, Ferula Assa Foetida has been suggested to eliminate seizures and also in Pakistan and Afghanistan it is used in food components. Its plant is distributed in East of Afghanistan and West of Iran (Taghi & Tehran, 1990; Moatar & Shams-Ardakani, 1999; Tabatabyi 1989). Ferula Assa Foetida has antispasmodic effects. It is used to eliminate the respiratory and gastrointestinal diseases with neural origins (Newall, Anderson & Phillipson, 1996) and is also prescribed for the treatment of inflammation, tremors and epilepsy in children (Gorgi & Madeja 2001). The most important substances in Ferula Assa Foetida, are sulfide compounds, Sesquiterpene compounds (Coumarin derivatives) and Monoterpenes including α-Pinene, β-,Pinene (With anti-epileptic properties) (Stefan et al., 2006), Phellandrene, Granyl acetate, α-terpineol, asaresinotannols and flavonoid compounds (with anti oxidant properties) and Luteolin (Dehpour et al., 2009).

In the researches performed on this plant, anticonvulsant, antioxidant and NO scavengering effects of this plant are mentioned (Sayyah et al., 2011). In this study we have investigated the anti-epileptic effects of Ferula Assa Foetida by evaluation of the antioxidant properties of this plant. In addition, by measuring the levels of NO as a free radical, MDA level as the most important indicator of lipid peroxidation, and SOD activity as an antioxidant enzyme in the PTZ-induced chemical kindling models, we examined the antioxidant properties of Ferula Assa Foetida.

2. Methods

2.1. Animals

In this experimental study, a total of60 male Albino mice weighing 25-30 g (Razi Institue, Iran) were randomly divided into six experimental groups including; 1- the control group, 2- PTZ-kindled mice, 3- the positive control group which received valproate (100 mg/kg) as anti-convulsant drug, 4-5 & 6- the treatment groups which received 25, 50 and 100 mg/kg; i.p. of Ferula Assa Foetida gum extract.

Ten mice per cage were kept in animal house of Shahed Medical University at temperature 21±2ºC and under light cycle of 12 h darkness and 12 h lights. The mice had free access to standard food and tap water ad libitum. The experimental protocol was approved by the Ethic Committee of Shahed University.

2.2. Kindling Method

All groups of mice except the control group were kindled by a total of 11 injections of PTZ (35 mg/kg; i.p.) every second day and in a period of 22 days. PTZ (Sigma) was dissolved in isotonic saline solution (NaCl 9/0%). The animals were considered for 30 minutes after the last drug injection; after an additional 30 minutes the mice were observed for lethality. On day 24, the challenge dose of PTZ (75 mg / kg) was injected to all of the kindled mice, to induce tonic-clonic seizures and perhaps death.

In the positive control group and treatment groups (groups 3, 4, 5 and 6) PTZ was administrated 30 minutes after the first treatment with valproate and different doses of Ferula Assa Foetida gum extract. The intensity of seizure (0-6 phases) in the kindling model for 30 minutes after PTZ injection was evaluated using the following scale (Eracovic et al., 2001). The scale introduces six phases as follows:

0: No response

1: Ear and facial twitching

2: Axial convulsive waves through the body

3: Myoclonic body jerks

4: Generalized clonic convulsions turning over into side position

5: Generalized convulsions with tonic extension episode and status epilepticus

6: Mortality

2.3. Preparation of Plant Hydro-Alcoholic Extract

Ferula Assa Foetida gum was provided from the local stores and was scientifically approved by the department of Botany of Shahed University. To prepare the hydro-alcoholic extract, using percolation method; 60 g of gum was grinded, and then the extract was obtained according to the Khalili method (Khalili et al., 2011; Atilla et al., 2006). The final concentration was 25%. However, the 25, 50 and 100 mg/kg extract doses were prepared from concentrated extract which were dissolved in saline.

2.4. Sample Preparation and Biochemical Assays

To evaluate the biochemical factors, after the 12th injection and behavioral observation, the mice were decapitated and their brains were quickly removed. The brains were washed two times in cold saline. They were placed in freezer (-30˚ C), in a glass bottle (for less than 10 hours). Then the brains were cut by scissors and were homogenized using four times ice-cold Tris-Hcl (50 mM, PH 7.4) buffer for 2 minutes at 5000 rpm. MDA and NO levels were measured at this phase. The homogenized solution was then centrifuged for 60 minutes at 5000×g to remove debris. The supernatant solution was then extracted with a mixture of ethanol/chloroform (a volume with ratio of 5:3). After centrifugation at 5000×g for 30 min, the clear upper layer (the ethanol phase) was taken and used for evaluation of the SOD activity. All experiments were carried out at +4˚ C (Oliver et al., 1990).

2.5. NO Measurement

Since nitric oxide is a highly unstable material that rapidly converts to nitrate (NO3-) and finally to nitrite (NO2-), the total nitrite amount using Griess method was used as an indicator of nitric oxide. Briefly, in this study nitrate in the samples was converted to nitrite by cadmium and followed by color development with Griess reagent (sulfanilamide and N-naphthyl ethylenediamine) in acidic medium. The absorbance was determined at 540 nm with a spectrophotometer (Ilhan, et al 2005).

2.6. SOD Enzyme Activity Evaluation

Total SOD activity was measured based on the Sun method. Supernatants of brain tissue samples were incubated with xantine and xanthine oxidase in potassium phosphate buffer (pH 7.8, 37˚C) for 40 min and NBT was added. Blue formazan was then monitored spectrophotometrically at 550 nm. The inhibition of NBT reduction to 50% maximum by SOD enzyme was obtained and wasdefined as 1 nitrite unit (NU) of SOD activity (Sun & Oberley, 1988).

2.7. MDA Evaluation

Measurement of malondialdehyde levels (thiobarbituric acid reactive substances, TBARS) is in accordance with a method in which MDA at 100° C and pH=2-3 reacts with thiobarbituric acid such that trichloroacetic acid and TBARS reagent have mixed with supernatant. After cooling on ice and centrifuging at 3000 rpm for 20 minutes, the supernatant light absorption was read at 532 nm (Oliver et al., 1990).

3. Statistical Analysis

The obtained results in this study were expressed as means ± S.E.M. Statistical analyses between experimental groups were carried out using repeated measurement of one way analysis of variance (ANOVA). The comparison between individual experimental groups was continued with complementary post-hoc Tukey test and p values less than 0.05 were considered as significant differences.

4. Results

4.1. Effect of Different Doses of Ferula Assa Foetida Gum Extract on the PTZ-Induced Seizure Intensity

Statistical analysis of results (as shown in figure 1) indicates that Ferula Assa Foetida gum extract 25mg/kg could reduce seizure intensity significantly only at the 6th and 7th injections as compared to the PTZ group)P< 0.05(. In addition, plant extract 50 mg/kg at the 6th, 7th and 9th injections was able to reduce seizure intensity significantly in comparison with PTZ group) P< 0.05(. Also, Ferula Assa Foetida gum extract 100 mg/kg in the 7th, 8th , and 9th injections, significantly reduced PTZ-induced seizure intensity as compared to

the PTZ group)P< 0.05(. Finally, the last injection in the challenge dose of Ferula Assa Foetida gum extract (100 mg/kg) was able to reduce seizure rate significantly along with valproate) P< 0.01(. It is noteworthy that, in the positive control group, valproate (100 mg/kg) has reduced seizure intensity in most of the periods significantly (P<0.05) relative to the PTZ group and had a more significant reducing effect in the challenge dose than in the other periods (P<0.001).

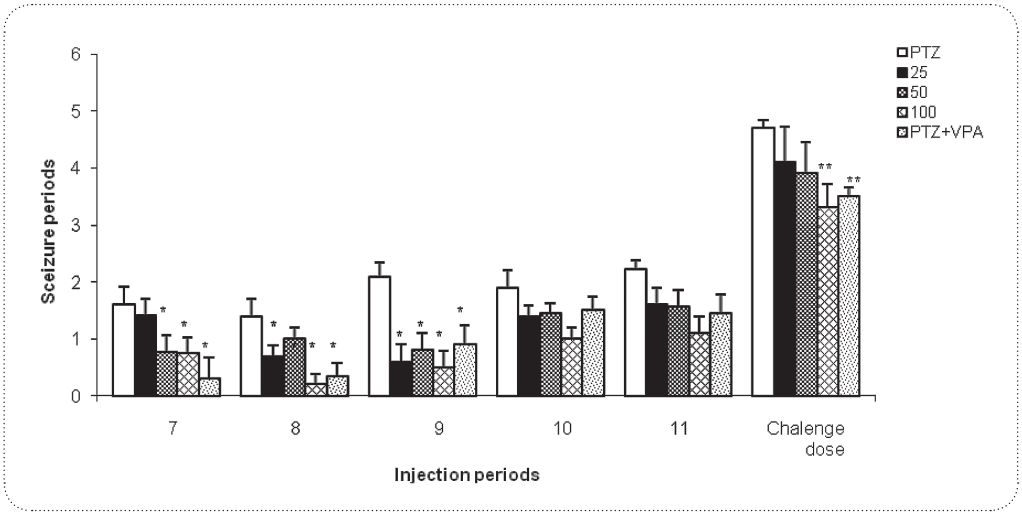

Figure 1. Effect of Ferula Assa Foetida pretreatment on the PTZ-induced kindling intensity. Ff shows Ferula Assa Foetida. *P < 0.05 and **P < 0.01 indicate significant differences as compared to PTZ-kindled group.

4.2. Effect of Different Doses of Ferula Assa Foetida Gum Extract on the Starting Time of Stage 5 of PTZ-Induced Seizure

As it is indicated in figure 2, Ferula Assa Foetida gum extract 100 mg/kg has shown a significant increase in seizure starting time as compared to PTZ group)P< 0.05(. In the rest of the treatment groups, significant differences were not observed.

4.3. Effect of Different Doses of Ferula Assa Foetida Gum Extract on State 5 Duration Time

As it is shown in figure 3, only pretreatmented of mice with Ferula Assa Foetida gum extract 100 mg/kg and valproate 100 mg/kg are able to reduce the period that the mice remain in phase 5 of seizure significantly (P<0.01 and P<0.05 respectively).

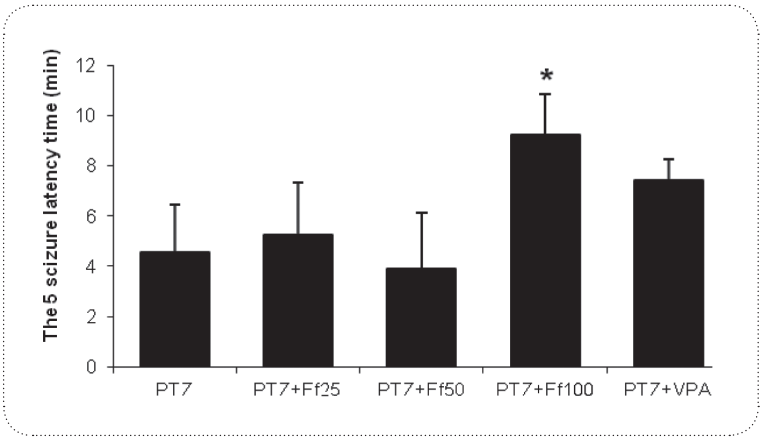

Figure 2. Effect of valproate (100 mg/kg) and three doses of Ferula Assa Foetida (25, 50 and 100 mg/kg) on the latency to the onset of stage 4 seizure. n=10 in each group. VPA and Ff indicate valproate and Ferula Assa Foetida respectively. *P < 0.05 indicate significant differences as compared to PTZ-kindled group

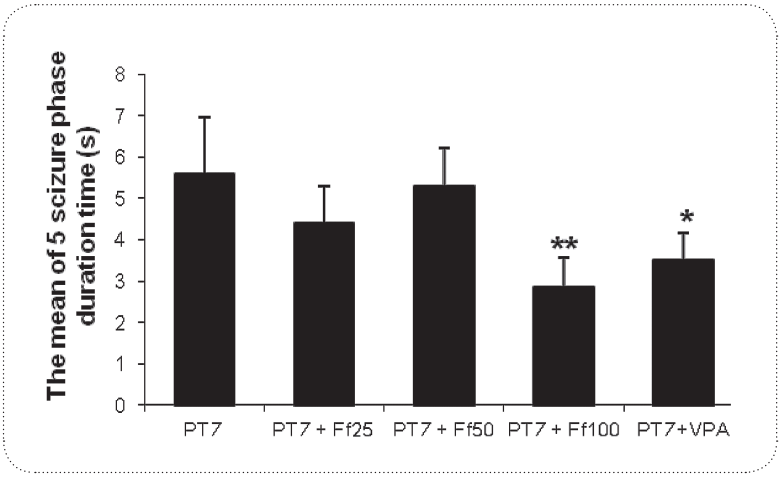

Figure 3. Effect of valproate (100 mg/kg) and three doses of Ferula Assa Foetida (25, 50 and 100 mg/kg) on the remaining time in the phase 5. n=10 in each group. VPA and Ff indicate valproate and Ferula Assa Foetida respectively. *P < 0.05 shows significant difference as compared to PTZ-kindled group.

4.4. Effect of Different Doses of Ferula Assa Foetida Gum Extract on the NO and MDA Values, and SOD Activity

A comparison among the effects of different doses of Ferula Assa Foetida gum extract, valproate and PTZ on the biochemical factors of brain tissue that are the common oxidative stress markers, is shown in table 1. As it is shown in the table, in the PTZ-kindled group, MDA level of the brain tissue has increased significantly relative to the control group)P< 0.05(and SOD level has shown a significant reduction in this group compared to the control group)P< 0.05(. In this group, however, the NO level did not show any significant change compared to the control group.

In the positive group which received valproate, the NO level showed a significant decrease compared to the control and PTZ groups (P< 0.05(. MDA levels in this group had significant reduction in comparison with the PTZ receiving group (P< 0.05(. However, valproate and different doses of plant extract were not able to induce any significant differences in SOD levels in the brain tissue in comparison with the PTZ and control groups.

In the experimental groups receiving different doses of plant extract, only Ferula Assa Foetida gum extract 50mg/kg induced significant reduction of the NO level compared with PTZ group)P< 0.05(. The mice which were pretreated with Ferula Assa Foetida gum extract 50 mg/kg and 100 mg/kg showed significant decrease of MDA levels compared to the PTZ group) P<0.05 and P< 0.01 respectively (. Ferula Assa Foetida gum extract 25 mg/kg did not make any significant differences of enzymes amounts compared with PTZ and control groups.

Brain levels of NO, MDA and SOD are compared in six groups. In each group n=10 and Ff indicates Ferula Assa Foetida * and # show significant differences as compared to control and PTZ-kindled groups respectively (P<0.05).

Table 1. The effect of valproate and three doses of Ferula Assa Foetida on the NO, MDA and SOD levels of brain tissue on the PTZ-kindled mice.

Groups	SOD (U)	MDA (nmol)	NO (µmol)
Control	0.127±0.004	17.69±1.89	0.527±0.027
PTZ	0.098±0.008 *	25.63±2.11 *	0.598±0.032
PTZ + Valproate	0.121±0.118	20.46±1.98 #	0.398±0.042 *
PTZ + Ff 25	0.123±0.014	23.51±1.19	0.497±0.056
PTZ + Ff 50	0.144±0.012	18.21±1.48 #	0.401±0.019 #
PTZ + Ff 100	0.146±0.014	15.29±1.33 # #	0.521±0.052

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5. Discussion

For centuries in the Iranian and Indian traditional medicine, Ferula Assa Foetida has been administered as an anti-seizure drug (Zargari, 1991). Additionally, in the western tradition of herbal medicine, it was used for the treatment of asthma, epilepsy, convulsions, and muscle cramps (Arky, 1996). In general, results of this research indicated that Ferula Assa Foetida gum extract has a lowering effect on seizure intensity and is able to enhance the seizure threshold. Our result is in consistence with previous studies that pointed to the anti-epileptic properties of this plant family in the PTZ model of epilepsy (Yajima, et al., 2000). All administrated doses of Ferula Assa Foetida gum extract could partly prevent the development of epilepsy. Ferula Assa Foetida gum extract at higher doses could significantly reduce the remaining period in the stage 5 of seizures. In addition, Ferula Assa Foetida gum extract 100 mg/kg postponed the starting time of phase 5 of the seizure. However, in a recent study it is reported that some species of Ferula did not suppress the PTZ-induced seizure (Bagheri, 2010). The discrepancy could rise from different parameters. The use of Ferula 300mg/kg in their experiment (which might be toxic), difference in the species of plant, the single dose of PTZ that they have used instead of chronic administration and the use of kindling in our experiment are some possible reasons for different results.

Rogawski and colleagues have pointed to this issue that PTZ causes epilepsy through the activation of glutamate receptors (NMDA) and stimulation of the calcium ions for entering into the nerve cells (Rogawski & Porter, 1995). Additionally, according to previous reports, PTZ-induced epilepsy (absence epilepsy) could be inhibited by T-type calcium current lowering drugs such as ethosuximide (Coulter, Hugenard & Prince, 1989). Researchers have also shown that family members of this plant could exert their analgesic effects through stimulation of opioid receptors in the central nervous system (Fazly Bazaz et al, 1997). It has also been proven recently that opioid receptor stimulation has an anti-seizure effect (Yajima et al., 2000). Since the opioid receptor agonists mainly affects calcium channels and inhibits calcium entry into nerve cells (Werz & Macdonald, 1984), probably the ferula family, through the stimulation of opioid receptors with inhibition of calcium entry into post-synaptic neurons, could reduce nervous stimulation in the central nervous system and exert inhibitory effects on seizure.

In the present study, the maximum anti-epileptic response of Ferula Assa Foetida gum extract in terms of suppressing seizure attacks, the latency of epileptic response, and the duration of the epileptic statues was related to the dose of 100 mg/kg. This dose is the most effective dose of the plant in this field and the more the doses reduce, the more the effects are lowered. Therefore, it seems that these effects are dose dependent. Of course this issue can be investigated more thoroughly by increasing the number of cases.

The generated oxidative stress in the brain is a common mechanism of cellular damage in many acute neurological attacks, such as seizure activity and diseases like Alzheimer's Disease (Oliver et al., 1990). Also membrane lipids are full of unsaturated fatty acids such as arachidonic acid and most of them are susceptible to lipid peroxidation process which leads to the destruction of the membrane and hindering its functions (Kim et al, 2000; Mandegary, Sayyah & Heidari, 2004). Of course, in the normal system body, the harmful effects of oxidative stress and free radical are controlled to some extent by antioxidant systems such as SOD enzyme (Freitas R, 2009). In epileptic patients serum levels of antioxidants are reduced and due to elevation of free radical levels, lipid peroxidation will be increased (Sudha, Rao & Rao, 2001). Ilhan and colleagues also reported that PTZ will lead to seizure through the induction of oxidative stress, and that anti-oxidant administration significantly reduces both oxidative stress and PTZ-induced seizure (Ilhan, 2005). It is also expressed in other resources that probably PTZ is the initiator of membrane structure lysis process and it causes the release of lipid peroxidases and free radicals (Obay, 2008).

In the present study in the PTZ receiving group, SOD enzyme levels as an antioxidant enzyme reduced and MDA levels as an indicator of lipid peroxidation showed a significant increase indicating the oxidative stress effects of PTZ administration. Thus, in accordance with other studies, our research indicates that the increment in free radicals and oxidative stress which is induced by PTZ is probably one of the causes of epilepsy (Khalili et al., 2011).

In previous studies by Sayyah and his colleagues, it was indicated that approximately seventy percent of the plant extracts contain α and β- Pinene (Sayyah et al., 2001). Existence of monoterpenes including α-Pinene and β-,Pinene (with anti-epileptic properties) (Stefan et al, 2006) is reported in Ferula Assa Foetida (Dehpour et al, 2009). It is also shown that Pinene analogs prevent idiopathic epilepsy in prone mice (Guzmán-Gutiérrez, 2012). Therefore, from this information one can conclude that the suppressing effect of the extract on the PTZ-induced seizure may be mediated by Pinene compounds. Previous studies have identified that sesquiterpenes are able to exert inhibitory effects on arachidonic acid-metabolizing enzymes and NO synthesis enzyme (as a free

radical). Regarding the existence of sesquiterpenes in the Ferula species (Dehpour et al, 2009) by which prevent the formation of metabolites than that caused by arachidonic acid metabolism in membranes, probably these plants act as antioxidants. In addition, flavonoid compounds with anti-oxidant properties are among the fractions of ferula plants and could be another candidate by which the anti-convulsant effect of ferula is occured.

NO is a molecule that is associated with regulation of neuronal stimulation ability and epileptic activity. Involvement of NO molecule in epilepsy and seizure making has been proven by numerous experiments via systemic injection of the NO synthetas inhibitor (Buisson et al., 1993). Furthermore, it has been shown that the NOS inhibitors act against acute seizures and progression of PTZ-induced kindling (Tsuda, Suzuki & Misawa, 1997). PTZ administration increases the NOS gene expression via activation of the calcium - calmodoline route in nerve cells and thus increases NO production in the brain (Swamy et al., 2010). The results of our study are also in consistence with this theory that NO levels are increased in PTZ receiving mice. NO increases cGMP in the cell, and the amino acid glutamate, as stimulatory neurotransmitter in the brain through increased cGMP levels causes increment of neuronal excitability (Oliveira et al., 1997). Taking together these evidences, one can conclude that additional NO in the brain increases cGMP that finally causes increment of neuronal activity and irritability, for providing an epileptic condition.

Our findings are also in consistence with previous studies that showed Ferula assa Foetida methanolic gum extract has NO scavengering properties (Dehpour et al., 2009) and probably the anticonvulsant effect of this extract is induced via NO reduction in mice tissue. Thus it seems that this plant is an effective plant in NO scavengering, and giving an absolute theory in this field needs more investigations.

In the body's normal physiological conditions, antioxidants defense system controls free radicals-induced tissue damage (Ilhan et al., 2006). Superoxide free radical is converted to hydrogen peroxide by the SOD enzyme. Thus, SOD enzyme protects cells against harmful superoxide radicals and the resulting oxidative stress. In this study, SOD enzyme activity in mouse brain tissue was significantly decreased in the PTZ group compared to the control group. Reduction of this antioxidant enzyme during seizure is probably due to excess consumption of this enzyme resulted from free radical production during PTZ-induced seizure. However, our plant could not scavenge the superoxide radical from the surface of the animal's brain via increasing the superoxide dismutase enzyme and had little effect in this regard.

MDA is also a lipid peroxidation marker that is caused by free radicals and therefore is an oxidation product (Ilhan et al., 2006). Significant increase of this index in PTZ group compared to the control group showed that during PTZ-induced seizures, free radicals are produced and neuronal membrane lipids suffer from oxidative stress induced peroxidation.

Considerable reduction of MDA levels in the plant extract treatment groups compared to the PTZ group implies that probably Ferula assa Foetida gum extract causes a decrease in oxidative damage and lipid peroxidation due to its antioxidant properties. In summary, according to the findings of present research, probably the lowering effects of hydro-alcoholic Ferula assa Foetida gum extracts on the PTZ-induced seizures is probably due to its antioxidant properties and decrease of oxidative stress.

References

Arky, R. (1996) Physicians' desk reference. Medical Economics Montvale. 50nd ed. New Jersey. 846.

Atilla, I., Mustafa, I., Suat, K.Y. (2006) Pentylenetetrazol-induced kindling seizure attenuated by Ginkgo biloba extract (EGb 761) in mice. Prog. Neuropsychopharmacol. Biol. Psychiatry, 30(8), 1504-1510.

Bagheri, S. M., Sahebkar, A., Gohari, A. R., Saeidnia, S., Malmir, M., Iranshahi, M. (2010) Evaluation of cytotoxicity and anticonvulsant activity of some Iranian medicinal Ferula species. Pharm. Biol, 48(3), 242-6.

Buisson, A., Lakhmeche, N., Verrecchia, C., Plotkine, M., Boulu, R. G. (1993) Nitric oxide: an endogenous anticonvulsant substance. NeuroReport., 4, 444-446.

Chauhan, A., Chauhan, V. (2006) Review of Oxidative stress in autism. Pathophysiology, 13, 171-181.

Coulter, D. A., Hugenard, J. R., Prince, D. A. (1989) Characterization of the ethosuximide reduction of low-threshold calcium current in thalamic neurons. Annals Neurology., 25, 582-593.

Dehpour, A. A., Ebrahimzade, M. A., Nabavi, S. F., Nabavi, S. M. (2009) Antioxidant activity of the methanol extract of Ferula assa foetida and its essential oil composition. Grasas Y Aceites, 60(4), 405-412.

Erakovic, V., Zupan, G., Varljen, J., Laginja, J., Simonic, A. (2001). Altered activities of rat brain metabolic enzymes caused bypentylenetetrazol kindling and pentylenetetrazol-induced seizures. Epilepsy Research 43, 165-173.

Evereklioglu, C., Turkoz, Y., Calis, M., Duygulu, F., Karabulut, A. B. (2004) Tumour necrosis factor a, lipid peroxidation and NO are increased and associated with decreased free-radical scavenging enzymes in patients with Weill Marchesani syndrome. Mediators of Inflammation, 13(3), 165-170.

Fazly Bazaz, B. S., Parsaei, H., Haririzadeh, G. (1997) Evaluation of antinociceptive and antimicrobial activities of galbanum plant (Ferula gummosa). Daru, 7, 1 -22.

Freitas, R. M. (2009) Investigation of oxidative stress involvement in hippocampus in epilepsy model induced by pilocarpine. Neurosci Lett., 462(3), 225-9.

Gorgi, A., Madeja, M. (2001) Lowering of the potassium concentration induces epileptiform activity in guinea pig hippocampal slices. Brain Res., 908, 130-139.

Guzmán-Gutiérrez, S. L., Gómez-Cansino, R., García-Zebadúa, J. C., Jiménez-Pérez, N. C., Reyes-Chilpa, R. (2012) Antidepressant activity of Litsea glaucescens essential oil: Identification of β-pinene and linalool as active principles. J Ethnopharmacol., 143(2), 673-9.

Ilhan, A., Iraz, M., Gurel, A., Armutcu, F., Kamilsi, S. (2005) Antiepileptogenic and antioxidant effects of Nigella sativa oil against pentylentetrazole-induced kindling in mice. Neuropharm., 49, 456-64.

Ilhan, A., Iraz, M., Kamisli, S., Yigitoglu, R. (2006) Pentylenetetrazol-induced Kindling seizure attenuated by Ginkgo biloba extract (Egb 761) in mice. Neuro-Psychopharmacology & Biological Psychiatry., 30, 1504-1510.

Khaleghi Ghadiri, M., Gorji, A. (2004) Natural remedies for impotence in medieval Persia. International Journal of Impotence Research, 16, 80-83.

Khalili M, Kiasalari Z, Roghani M, Azizi Y. (2011) Anticonvulsant and antioxidant effect of hydroalcoholic extract of Cyperus rotundus rhizome on pentylentetrazole-induced kindling model in male mice. Journal of Medicinal Plants Research, 5(7): 1140-1146.

Kim, H. C., Jhoo, W. K., Bing, G., Shin, E. J., Wie, M. B., Kim, W. K., Ko, K. H. (2000) Phenidone prevents kainate-induced neurotoxicity via antioxidant mechanisms. Brain Res, 874(1): 15-23.

Levav, I., Stephenson, C., Theodore, W. H. (1999) Epilepsy in Latin America and the Caribbean: a survey on needs and resources. Pan Am J Public Health., 6(5), 342-345.

Mandegary, A., Sayyah, M., Heidari, M. R. (2004) Anti-nociceptive and Anti-inflammatory activity of the seed and root extracts of Ferula Gummosa Boiss in mice and rats. Daru., 12(2), 58-62.

McNamara, J. O. (1999) Cellular and molecular basis of epilepsy. J.Neurosci., 14, 3413-3425.

Moatar, F., Shams, Ardakani, M. (1999) Instructions for herbal therapy. Tehran. The Academy of Med Sci, 2, 73-75.

Newall, C.A., Anderson, L. A., Phillipson, J. D. (1996) Herbal medicines. London: The pharmaceutical press., 96, 38-9.

Obay, B. D., Tademir, E., Tümer, C., Bilgin, H. M., Atmaca, M. (2008) Dose dependent effects of ghrelin on pentylenetetrazole-induced oxidative stress in a rat seizure model. Peptides., 29(3), 448-455.

Oliver, C. N., Starke-Reed, P. E., Stadtman, E. R., Lin, G. J., Correy, J. M., Floyd, R, A. (1990) Oxidative damage to brain proteins, loss of glutamine synthetase activity and production of free radicals during ischemia/reperfusion induced injury to gerbil brain. Proc Natl Acad Sci USA., 87, 5144–7.

Oliveira, P. R., Del-Bel, E. A., Oliveira, J. A. C., Mishra, P. K., Jobe, P. C., Carcia- Cairasco N. (1997) Anticonvulsant and proconvulsant roles of nitric oxide in experimental epilepsy models. Braz J Med Bio Res., 30(8), 971- 979.

Rogawski, M. A., Porter, R. J. (1995) Antiepileptic drugs and pharmacological mechanisms and clinical efficacy with consideration of promising developmental stage compounds. Pharmacological Review., 42, 223-286.

Rukmini, M. S., D'Souza, B., D'Souza, V. (2004) Superoxide dismutase and catalase activities and their correlation with malondialdehyde in schizophrenic patients. Indian Journal of Clinical Biochemistry., 19(2), 114-118.

Sayyah, M., Kamalinejad, M., Bahrami Hidage, R., Rustaiyan, A. (2001) Antiepileptic Potentional and Composition of the Fruit Essential Oil of Ferula Gummosa boiss. Iranian Biomed J., 2, 69-72.

Sogut, S., Zoroglub, S. S., Zyurtc, H., Yılmazd, H. R., Zugurluc, F., Sivaslıe, E. (2003) Changes in nitric oxide levels and antioxidant enzyme activities may have a role in the pathophysiological mechanisms involved in autism. Clinica Chimica Acta., 331, 111-117.

Stefan, H., Lopes da silva, F. H., Loscher, W., Schmidt, D., Perucca, E., Brudie, M. (2006) J.Epilptogenesis and rational therapeutic strategies. Aeta Neural Scand., 113, 139-155.

Sudha, K., Rao, A. V., Rao, A. (2001) Oxidative stress and antioxidants in epilepsy. Clin. Chem., 303, 19-24.

Sun, Y., Oberley, L. W., Li, Y. (1988) A simple method for clinical assay of superoxide dismutase. Clinical Chemistry., 34, 497-500.

Swamy, M., Salleh, M. J., Sirajudeen, K. N., Yusof, W. R., Chandran, G. (2010) Nitric oxide (NO), citrulline - no cycle enzymes, glutamine synthetase and oxidative stress in anoxia (hypobaric hypoxia) and reperfusion in rat brain. Int. J. Med. Sci., 7(3), 147-154.

Tabatabyi, S. M. (1989) In translate of Alhavi of Zakaria Razi. Tehran, Mashhad Med Sci University publication., 1, 127-153.

Taghi, M. M. (1990) Tehran. In translate of Genuine authority of Ansari Shirazi. Razi med Distribution Company., 2, 136.

Theodore, W. H., Fisher, R. (2007) Brain stimulation for epilepsy. Acta Neurochir Supp., 97(2), 261-272.

Tsuda, M., Suzuki, T., Misawa, M. (1997) Aggrivation of DM-CM-induced seizure by nitric oxide synthase inhibitors in mice. Life Sci., 60, 339- 343.

Tuszkiewicz - Misztal, E., Opoka - Winiarska, V., Postêpski, J. (2000) Significance of dietary antioxidants for child proper development and health. Pediatr Pol., 75, 359–366.

Werz, R. L., Macdonald, M. A. (1984) Dynorphin reduces voltage – dependent calcium conductance of mouse dorsal root ganglion neurons. Neuropept., 5, 253-6.

Yajima, Y., Naritaa, M., Takahashi-Nakano, Y., Misawa, M., Nagase, H., Mizoguchi, H. (2000) Effects of differential modulation of μ-, δ- and κ-opioid systems on bicuculline-induced convulsions in the mouse. Brain Res., 862, 120-26.

Zamani, S. (1999) Medicinal plants. Tehran Med. Sci. University, 2, 5-8.

Zargari, A. (1991) Medicinal plants. Tehran Med. Sci. University, 5, 231-226.

Stroke Modifies Drug Consumption in Opium Addicts: Role of the Insula

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ABSTRACT

Introduction: Addiction imposes a large medical, social and economic burden on societies. Currently, there is no effective treatment for addiction. Our struggle to decipher the different mechanisms involved in addiction requires a proper understanding of the brain regions which promote this devastating behavior. Previous studies have shown a pivotal role for insula in cigarette smoking. In this study we investigated the change in opium consumption after CVA.

Methods: This study took place in three referral academic hospitals affiliated to Tehran University of Medical Sciences. Patients who suffered a CVA and were addicted to opium were recruited during their hospitalization or visit to the neurology clinic in this study. Age, sex and the route and mean amount of opium use of each patient before CVA and 1, 3 and 6 months post-CVA was asked using a questionnaire. The patients were divided into three groups based on the location of brain ischemia (insula, basal ganglia and non-insula non-basal ganglia group).

Results: Seventy five percent of the patients with ischemia of the insula changed the route or amount of opium use after CVA and 37.5% of them stopped opium use after CVA. These values were significantly higher than patients with non-insula non-basal ganglia ischemia (p values 0.005 and 0.03 for change in route or amount and stopping opium use, respectively). This was not true in patients with ischemia of the basal ganglia. Younger patients were more likely to change the route or amount of opium use and stop opium use after CVA (p values 0.002 and 0.026, respectively).

Discussion: The results of the present study indicate a possible role for the insula in opium addiction, especially in younger individuals.

Key Words:
Stroke,
Addiction,
Opium.

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1. Introduction

Opium contains morphine and codeine and its narcotic and analgesic properties are attributed to these compounds.(Reddy, Suresh, Jayashanker, Rao, & Sarin, 2003) It is a widely used addictive substance in Asian countries including Iran.(Mokri, 2002) Opium is recreationally used by inhalation, oral intake or enema.(Alemi, 1978; Paoli, Greenfield, Charles, & Reuter, 2009) Illicit drug abuse imposes a large social, economic and medical burden on the society.(Ghotbi & Tsukatani, 2007; Rehm et al., 2009)

Much endeavor has been devoted to unravel the mysteries of addictive behavior. Regarding this fact, a great focus has been placed on exploring different brain regions implicated in addiction. Studies on psychostimulants constitute the great proportion of research in this field.(Degoulet, Rostain, Abraini, & David, 2011; Veeneman, Broekhoven, Damsteegt, & Vanderschuren, 2011) However, a gap exists in the field of opiates.

Classically, the ventral tegmental area and nucleus accumbens (NAcc) have been considered to play the key role in addiction.(Bradberry & Roth, 1989; Nisell, Nomikos, & Svensson, 1994) However, more recent research revealed that these regions are not the sole promoters of addictive behavior. Apart from the NAcc, other constituents of the basal ganglia have also been suggested to have an important role in the formation of addictive behavior.(Everitt & Robbins, 2005) Lately, there has been a great focus on the insula as a probable neuroanatomical substrate of addiction.(Naqvi & Bechara, 2009; Naqvi, Rudrauf, Damasio, & Bechara, 2007)

Studies on addicted patients who suffer from a cerebrovascular accident (CVA) can provide a better understanding of different brain regions involved in addiction. Naqvi et al. studied 69 cigarette smokers with CVA. Their findings implicated a pivotal role for the insula in addiction to cigarette. In the present study we assessed the change in amount of opium consumption by addicted individuals with CVA, with regard to the location of their ischemia.

2. Methods

This study was undertaken in three hospitals of Tehran University of Medical Sciences. Data were collected at neurology clinics. We interviewed patients who had been hospitalized during February 2009 to May 2011 with a diagnosis of ischemic CVA and had a previous history of opium consumption documented during their hospitalization period or their follow up visit to the hospital's neurology clinic. We only included patients with ischemic CVAs to insure that the investigated groups are as homogeneous as possible, and therefore, those with hemorrhagic CVAs were not studied. Only patients who had been using opium for a period of at least one year prior to CVA were included in the study. Patients with aphasia or amnesia, those with physical disability which hampered drug use (e.g. debilitating movement disorder and blindness) and those who were receiving methadone maintenance therapy were excluded. Written informed consents were obtained from patients who agreed to participate in the study. Participants were assured that

Table 1. Different anatomical regions in each group of CVA

Insula	Right or left insula ischemia
Basal Ganglia	periventricular , thalamus , basal ganglia and pons
	parietal lobe and basal ganglia
	lacunar
	head of caudate
Non Insular-non Basal Ganglia	lacunar ischemia of periventricular white matter , loop Myer , centrum semiovale
	left medullary infarction
	tempor-occipital
	temporal uncus
	frontal lobe and precentral gyrus
	temporo-parietal
	parieto-occipital
	hippocampus
	temporal, frontal, parietal lobes in cortical and white matter
	pons , medulla and corona radiata

the data would be kept confidential. This study was performed in accordance with the Declaration of Helsinki and it was also approved by the ethical review committee of Tehran University of Medical Sciences.

We used a checklist during the interviews. Sex, age at lesion onset, amount and route of opium consumption before CVA and at different time points after CVA (1, 3 and 6 months post-CVA) were documented. Since most of the patients referred to the neurology clinic about one month after discharge, they had to be contacted later to determine their opium consumption 3 and 6 months after CVA. At the end of the interview open questions were used to compare the financial status of the participants before and after CVA to rule out inability to afford opium as a reason for decrease in opium consumption. Brain MR Images and CT scans of the patients were obtained shortly after the initial interview (a little over a month after CVA). The images were examined by three different expert neuroradiologists to localize the site of the lesions. The neuroradiologists were unaware of the mean amount of opium used by the patients. We divided the patients into three groups based on the location of their lesions. These groups were the insula group, the basal ganglia group and the non-insula non-basal ganglia group.

The mean amount of opium consumption (grams per day) was calculated for each patient based on the data collected by the questionnaires. Patients were grouped based on the location of their lesions. SPSS version 13 (Chicago, IL, USA) was used to analyze the data. We used the Shapiro Wilk test to assess normality of the data. The Friedman's test was used to compare mean opium consumption before CVA and at different time points after CVA. We used a method described by Conover as the post hoc test for Friedman's test.(Conover, 1980) Mann Whitney U and Kruskal Wallis tests were used to compare mean amount of opium consumption and the percentage of reduction in opium use at different time points after CVA in different groups of patients. We used a binary logistic regression to investigate the presence of a relation between the location of brain lesions and cessation of opium consumption or change in route or mean amount of opium consumption after CVA. Age at lesion onset was entered as a covariable in this analysis.

3. Results

As mentioned earlier in the methods section, patients with post-CVA physical disabilities which hampered drug use were not included in the study. We interviewed 47 patients and all interviewed patients responded to all

Table 2. Amount of opium consumption before and after CVA in patients with insula and basal ganglia ischemia.

Anatomical location of stroke	Estimated amount of opium consumption (grams per day)			
	before CVA	1 month post-CVA	3 months post-CVA	6 months post-CVA
Insula	1.0	0.33	0.33	0.33
	0.33	0.33	0.33	0.33
	0.5	0.5	0.5	0.5
	1.5	0.33	0.33	0.33
	0.5	0.5	0.5	0.5
	0.33	0.0	0.0	0.0
	0.33	0.0	0.0	0.0
	0.5	0.0	0.0	0.0
Basal ganglia	1.5	1.5	1.5	1.5
	1.0	1.0	1.0	1.0
	3.0	0.75	0.75	1.0
	2.0	2.0	2.0	2.0
	1.5	1.0	1.0	1.0
	2.0	2.0	2.0	2.0
	0.33	0.0	0.0	0.0
	1.0	1.0	1.0	1.0
	0.5	0.0	0.0	0.0

questions. Two patients were excluded from the study to eliminate change in financial status after CVA as a confounding factor, since they stated that they lost their jobs after CVA. Another patient was excluded because of post-CVA blindness. The remaining patients were included in the study (40 males and 4 females). Their age ranged from 43 to 89 with a mean of 62 and a standard deviation of 9. The mean age of patients in the insula, basal ganglia and non-insula non-basal ganglia groups were 61.5 ± 1.6, 61.2 ± 2.0 and 62.6 ± 2.1, respectively, which were not significantly different from each other (data represented as mean ± S.E.M.). Interview sessions were discussed and arranged with the patients during their first interview. All 44 patients were interviewed in determined dates to evaluate their drug consumption habit in 3 and 6 months post CVA. Subsequent interpretations of their MRI revealed anatomic location of lesion, summarized in table-1. None of the patients in this study referred to any rehabilitation centers.

Seven patients (15.9%) suffered an ischemia of the insula. Eight patients (18.2%) had an ischemia in basal ganglia, one patient (2.2%) with infarction in both insula and basal ganglia, data of whom are presented in table-2, and one patient (2.2%) with hippocampus injury. Twenty seven patients (61.4%) had brain ischemia involving other brain regions. The patient who suffered a hippocampus infarction was grouped along with patients of the non-insula non-basal ganglia group. This patient didn't change the amount or route of opium use after CVA.

Seventeen patients (38.6 ± 7.2%) changed the amount or route of opium use after CVA. One of these patients had both an insular and a basal ganglia lesion. Five of them had an insular lesion without involvement of the basal ganglia and four of them had a basal ganglia lesion without involvement of the insula. The seven remaining patients who changed their route or amount of opium use had lesions in brain regions other than the insula or basal ganglia. Mean age of patients who did not change the route or amount of drug use was 66.1 ± 1.7, while patients who changed the route or amount of drug use had a mean age of 56.3 ± 1.6. Patients with infarctions of the insula were more likely to change the amount or route of drug use after CVA (p value = 0.005, odds = 32.8). However, patients with lesions in the basal ganglia did not have a higher chance of changing the route or amount of opium use after CVA in comparison to patients of the non-insula non-basal ganglia group (p value = 0.096). In addition, older patients had a lower probability of changing the amount or route of drug use after CVA (p value = 0.002, odds =0.80). Five out of six (83 ± 16%) patients with ischemia in the right insula changed their route or amount of opium consumption. We excluded the 2 patients with ischemia in the left insula and repeated the same analysis. As before, right insular ischemia significantly affected change in route or amount of opium consumption (p value = 0.01, odds = 36.15).

Eight patients stopped opium use after CVA and remained abstinent up to 6 months post-CVA. One of them had ischemia in both insular and basal ganglia, two had an ischemia in the insula without involvement of the basal ganglia and two with ischemia in the basal ganglia without involvement of the insula. The mean age of patients who stopped opium use after CVA was 55.9 ± 2.4 while mean age of those who did not stop opium use was 63.7 ± 1.5. Patients with ischemia in insula were more likely to stop opium consumption after CVA and remain abstinent in comparison to patients of the non-insula non-basal ganglia group (odds = 30.86, p value = 0.03). However, the analysis did not yield a significant p value when patients of the basal ganglia and the non-insula non-basal ganglia groups were compared (p value = 0.109). Age inversely correlated with the probability of stopping opium consumption (p value = 0.026, odds = 0.81). Three out of

Table 3. Comparison of the percentage of reduction in opium consumption in different groups (data are presented as mean ± SEM).

Group	Percent of reduction in opium consumption 1 month post-CVA	Percent of reduction in opium consumption 3 month post-CVA	Percent of reduction in opium consumption 6 month post-CVA
Insula	55.6 ± 16.8	55.6 ± 16.8	55.6 ± 16.8
Basal Ganglia	34.2 ± 14.9	34.2 ± 14.9	33.3 ± 14.7
non-Insula non-Basal Ganglia	12.5 ± 5.4	14.3 ± 5.8	14.9 ± 5.8
All Patients	24.5 ± 5.8	25.6 ± 5.9	25.8 ± 5.9

six (50 ± 22%) patients with ischemia in the right insula stopped opium use after CVA. The same analysis was repeated after excluding the two patients with ischemia in the left insula. As before, ischemia in the right insula was associated with stopping opium consumption after CVA (p value = 0.022, odds = 31.3).

The mean amount of opium used by the patients in different groups did not follow a normal distribution. The amount of opium use changed significantly after CVA in all three groups in our study (p values: 0.002, 0.012 and 0.002 for insula, basal ganglia and non-insula non-basal ganglia groups, respectively). The post hoc analysis revealed that mean opium consumption at 1, 3 and 6 months post CVA was significantly lower than mean opium consumption before CVA in all 3 groups of patients (p value < 0.05).

A Kruskal Wallis analysis revealed that the mean amount of opium use of patients of the insula, basal ganglia and non-insula non-basal ganglia groups were significantly different at all time points of the study (p values 0.03, 0.006, 0.006 and 0.008 for before CVA, 1 month post-CVA, 3 months post-CVA and 6 months post-CVA, respectively). Mann Whitney U test with a Bonferroni correction for multiple comparisons showed that patients with insular infarction used a significantly lower amount of opium before CVA, 1 month post-CVA, 3 months post-CVA and 6 months post-CVA in comparison to patients with non-insula non-basal ganglia infarctions (p values 0.033, 0.003, 0.003 and 0.003, respectively).

Since the mean amount of opium use before CVA differed between the three groups, we decided to compare the percentage of decrease in the amount of opium consumption between the groups instead of comparing the absolute amount of opium use at different time points between the groups (Table 3). The percentage of decrease in the amount of opium consumption in different groups was not normally distributed. Kruskal Wallis analysis revealed that there was a statistically significant difference between the percentage of decrease in the amount of opium consumption between the three groups (p values 0.033, 0.39 and 0.38 at 1, 3 and 6 months post-CVA, respectively). A Mann Whitney U test with a Bonferroni correction for multiple comparisons showed that patients with ischemia in insula had a higher percentage of reduction in opium use in comparison to patients with non-insula non-basal ganglia ischemia (p values 0.036, 0.039 and 0.036 at 1, 3 and 6 months post-CVA, respectively). However, this was not true for patients with ischemia in basal ganglia (p values 0.40, 0.45 and 0.52 at 1, 3 and 6 months post-CVA, respectively).

4. Discussion

Results of this study show that patients with ischemia in the insula were more likely to decrease or stop opium consumption after CVA. The present study provides further evidence for the role of insula in addiction. Several lines of evidence support the fundamental role of the insula in addiction to various compounds. Naqvi et al. reported 69 cigarette smokers with lesions in different brain regions. In accordance with our study, they found that patients with insular injury (especially right insular injury) had a higher probability of stopping smoking in comparison to patients with lesions in other brain regions. (Naqvi, et al., 2007) Furthermore, it has been shown that inactivation of the insula by lidocaine disrupts amphetamine induced place preference in rats.(Contreras, Ceric, & Torrealba, 2007) Additionally, schizophrenics (a population prone to cigarette addiction) and cocaine abusers have a reduced insula grey matter.(Crespo-Facorro et al., 2000; Franklin et al., 2002) This implicates that insula dysfunction might predispose individuals to addiction. (Naqvi & Bechara, 2009) Naqvi and Bechara suggest that insula plays an essential role in addiction by providing conscious pleasure, causing cue-induced urges and biasing decision making processes towards drug use. (Naqvi & Bechara, 2010) Our study is the first to provide evidence for the role of insula in addiction to opiates.

Craig proposed that insula receives sensory inputs from various parts of the body which are relevant to maintaining homeostasis, e.g. pain, taste, palpation, heartbeat, temperature, visceral senses from the respiratory and GI tract. Craig referred to these sensory inputs as interoception.(Blomqvist, Zhang, & Craig, 2000; Craig, 2002, 2004) The interoceptive inputs to insula, give rise to conscious emotions, i.e. the knowledge of how we are feeling. Considering the foregoing facts, Naqvi explains that the interoceptive effects of drugs which depend on the drug-use ritual (snorting, smoking, injecting etc) are aversive in nature but become pleasurable when drug use is continued because of being associated with reward. Another way of saying this is that the addicted individual learns to enjoy the interoceptive effects of drugs whether or not they are accompanied by the drug.(Naqvi & Bechara, 2009)

According to Naqvi et al. patients who suffer CVA are more concerned about their health. Thus a great proportion of cigarette smokers in their study quit smoking after CVA, irrespective of the location of their brain lesions. (Naqvi, et al., 2007) This might explain why the mean amount of opium use decreased in all three groups of patients (insula, basal ganglia and non-insula non-basal

ganglia group) after CVA in our study. Indeed, previous studies have shown that onset of smoking-related diseases (e.g. myocardial infarction, CVA and etc.) greatly increases the likelihood of smoking cessation.(Twardella et al., 2006) Notwithstanding the forgoing, patients with insular ischemia had a significantly higher percentage of reduction in the amount of opium use in comparison to patients with non-insula non-basal ganglia infarctions at different time points after CVA, indicating a key role for the insula in opium addiction.

Among all the various brain systems implicated in addiction by far the most notorious is the mesolimbic dopaminergic system. The neurons of this system arise from the brainstem ventral tegmental area and have dopaminergic projections to the NAcc. Today there is a consensus that the motivation for continued use of nearly all drugs of abuse is caused, at least in part, by their ability to release dopamine from the mesolimbic system.(Everitt & Robbins, 2005; Naqvi & Bechara, 2009) Although addiction is initially a goal directed act, it ultimately evolves into a stimulus-response (habit) behavior.(Everitt & Robbins, 2005) The dorsolateral striatum is thought to be responsible in maintaining the drug seeking habit during prolonged periods of drug absence. (Vanderschuren, Di Ciano, & Everitt, 2005) These findings have led to the belief that the striatum, as a whole, participates in the initiation and persistence of addictive behavior.(Everitt & Robbins, 2005) To assess the role of the striatum in opium addiction we studied patients with infarctions involving the basal ganglia and found no association between ischemia in basal ganglia and cessation/reduction of opium use after CVA. Although there is the possibility that opium addiction is independent of the striatum, it is also possible that bilateral basal ganglia infarction is required to influence opium use. The finding that bilateral pharmacological inactivation of the dorsal striatum is required to abrogate drug seeking behavior supports this idea.(Vanderschuren, et al., 2005) Another explanation for such an unexpected result could be the small sample size in our study. Further studies are required to elucidate the role of the basal ganglia in opium addiction.

Apart from its role in the formation of new memories, the hippocampus has a part to play in addictive behavior. When an individual confronts a previously experienced context of drug consumption, glutaminergic hippocampal projections to the NAcc are activated. This activation reinforces drug seeking behavior.(Everitt & Robbins, 2005; Nestler, 2001) Since hippocampal injuries alter locomotor activity (Caine, Humby, Robbins, & Everitt, 2001) it is likely that these contextual memories control the individual's motivation to seek drugs.(Everitt & Robbins, 2005) Previous studies emphasize on the role of the ventral hippocampus in addictive behavior.(Caine, et al., 2001) Only one patient had hippocampal infarction in our study. The amount of opium used by this patient did not change after CVA. This might be due to the location of the ischemia, since it involved only the posterior part of the hippocampus, sparing the ventral hippocampus.

Younger patients in our study were more likely to quit opium use after CVA. Although psychological motivations (i.e. holding addiction responsible for stroke in an early age and trying to avoid further drug use) could be an explanation for this finding, but this might also indicate that neural circuits of opium addiction change with aging. It has been shown that age affects mu-opioid receptor binding in many regions of the human brain. (Zubieta, Dannals, & Frost, 1999) Moreover, D2 dopamine receptors, which have been suggested to play a key role in formation of addictive behavior,(Noble, 2000) are reduced in older individuals.(Volkow et al., 1996) Contrary to opium addicts, younger cigarette smokers are less likely to quit smoking after CVA.(Bak et al., 2002) More research needs to be done to address this issue.

An unanticipated finding in our study was that individuals with ischemia in the insula consumed less opium before CVA and at different time points after CVA in comparison to individuals with non-insula non-basal ganglia ischemia. One explanation for this finding is that opium might make neurons of the insula more resilient against ischemia. A number of studies provide data in support of this assumption. It has been demonstrated that kappa opioid receptor agonists possess neuroprotective properties and decrease the infarction size in animal models of CVA. Interestingly, these opioid agonists do not cause cerebral blood flow alteration and probably act at the neuronal level. Moreover, this effect seems to be region specific, since it doesn't affect all brain regions. (Mackay, Kusumoto, Graham, & McCulloch, 1993) In another study, it was shown that endogenous opioids and exogenously administered morphine reduce the volume of infarction in an animal model of CVA.(Zhou et al., 2011) These findings might explain why patients who consumed higher amounts of opium were less likely to suffer insular infarctions. On the other hand, another explanation for this finding could be that individuals with more active insular cortices experience the same amount of desirable interoception with lower amounts of drugs. (Naqvi & Bechara, 2009) For this reason, these individuals routinely consume lower amounts of drugs than other addicts, yet they too experience the "high" feeling. Nevertheless, a more active insula might be more susceptible

to ischemic insults.(Saleh, Connell, Legge, & Cribb, 2004) More research needs to be done before claiming such assumptions.

One of the major shortcomings of our study is that we allocated all the patients with infarctions involving different parts of the basal ganglia into one single group. For instance, patients with strokes involving the amygdala were grouped along with those who had NAcc infarctions. Moreover, other locations which have been proposed to have a role in addiction, such as the orbitofrontal cortex, were not evaluated in our study.

In essence, the results of the present study are in support of previous studies suggesting a key role for the insula in addiction. In contrast to a previous study in cigarette smokers which reported that younger individuals are less likely to quit smoking after CVA, older patients in our study continued to use the same amount of opium after CVA. Additionally, patients with insular infarction in our study consumed less opium both before and after CVA.

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References

Alemi, A. A. (1978). The iceberg of opium addiction. An epidemiological survey of opium addiction in a rural community. Drug Alcohol Depend, 3(2), 107-112.

Bak, S., Sindrup, S. H., Alslev, T., Kristensen, O., Christensen, K., & Gaist, D. (2002). Cessation of smoking after first-ever stroke: a follow-up study. Stroke, 33(9), 2263-2269.

Blomqvist, A., Zhang, E. T., & Craig, A. D. (2000). Cytoarchitectonic and immunohistochemical characterization of a specific pain and temperature relay, the posterior portion of the ventral medial nucleus, in the human thalamus. Brain, 123 Pt 3, 601-619.

Bradberry, C. W., & Roth, R. H. (1989). Cocaine increases extracellular dopamine in rat nucleus accumbens and ventral tegmental area as shown by in vivo microdialysis. Neurosci Lett, 103(1), 97-102.

Caine, S. B., Humby, T., Robbins, T. W., & Everitt, B. J. (2001). Behavioral effects of psychomotor stimulants in rats with dorsal or ventral subiculum lesions: locomotion, cocaine self-administration, and prepulse inhibition of startle. Behav Neurosci, 115(4), 880-894.

Conover, W. J. (1980). Practical nonparametric statistics (2d ed.). New York: Wiley.

Contreras, M., Ceric, F., & Torrealba, F. (2007). Inactivation of the interoceptive insula disrupts drug craving and malaise induced by lithium. Science, 318(5850), 655-658.

Craig, A. D. (2002). How do you feel? Interoception: the sense of the physiological condition of the body. Nat Rev Neurosci, 3(8), 655-666.

Craig, A. D. (2004). Distribution of trigeminothalamic and spinothalamic lamina I terminations in the macaque monkey. J Comp Neurol, 477(2), 119-148.

Crespo-Facorro, B., Kim, J., Andreasen, N. C., O'Leary, D. S., Bockholt, H. J., & Magnotta, V. (2000). Insular cortex abnormalities in schizophrenia: a structural magnetic resonance imaging study of first-episode patients. Schizophr Res, 46(1), 35-43.

Degoulet, M., Rostain, J. C., Abraini, J. H., & David, H. N. (2011). Short-term development of behavioral sensitization to amphetamine requires N-methyl-D-aspartate- and nicotinic-dependent mechanisms in the nucleus accumbens. Addict Biol.

Everitt, B. J., & Robbins, T. W. (2005). Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. Nat Neurosci, 8(11), 1481-1489.

Franklin, T. R., Acton, P. D., Maldjian, J. A., Gray, J. D., Croft, J. R., Dackis, C. A., et al. (2002). Decreased gray matter concentration in the insular, orbitofrontal, cingulate, and temporal cortices of cocaine patients. Biol Psychiatry, 51(2), 134-142.

Ghotbi, N., & Tsukatani, T. (2007). The Economy of Opium and Heroin Production in Afghanistanand Its Impact on HIV Epidemiology in Central Asia. KIER Discussion Paper, 635.

Mackay, K. B., Kusumoto, K., Graham, D. I., & McCulloch, J. (1993). Effect of the kappa-1 opioid agonist CI-977 on ischemic brain damage and cerebral blood flow after middle cerebral artery occlusion in the rat. Brain Res, 629(1), 10-18.

Mokri, A. (2002). Brief overview of the status of drug abuse in Iran. Arch Iranian Med, 5(3), 184-190.

Naqvi, N. H., & Bechara, A. (2009). The hidden island of addiction: the insula. Trends Neurosci, 32(1), 56-67.

Naqvi, N. H., & Bechara, A. (2010). The insula and drug addiction: an interoceptive view of pleasure, urges, and decision-making. Brain Struct Funct, 214(5-6), 435-450.

Naqvi, N. H., Rudrauf, D., Damasio, H., & Bechara, A. (2007). Damage to the insula disrupts addiction to cigarette smoking. Science, 315(5811), 531-534.

Nestler, E. J. (2001). Neurobiology. Total recall-the memory of addiction. Science, 292(5525), 2266-2267.

Nisell, M., Nomikos, G. G., & Svensson, T. H. (1994). Systemic nicotine-induced dopamine release in the rat nucleus accumbens is regulated by nicotinic receptors in the ventral tegmental area. Synapse, 16(1), 36-44.

Noble, E. P. (2000). Addiction and its reward process through polymorphisms of the D2 dopamine receptor gene: a review. Eur Psychiatry, 15(2), 79-89.

Paoli, L., Greenfield, V. A., Charles, M., & Reuter, P. (2009). The global diversion of pharmaceutical drugs. India: the third largest illicit opium producer? Addiction, 104(3), 347-354.

Reddy, M. M., Suresh, V., Jayashanker, G., Rao, B. S., & Sarin, R. K. (2003). Application of capillary zone electrophoresis in the separation and determination of the principal gum opium alkaloids. Electrophoresis, 24(9), 1437-1441.

Rehm, J., Mathers, C., Popova, S., Thavorncharoensap, M., Teerawattananon, Y., & Patra, J. (2009). Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders. Lancet, 373(9682), 2223-2233.

Saleh, T. M., Connell, B. J., Legge, C., & Cribb, A. E. (2004). Estrogen attenuates neuronal excitability in the insular cortex following middle cerebral artery occlusion. Brain Res, 1018(1), 119-129.

Twardella, D., Loew, M., Rothenbacher, D., Stegmaier, C., Ziegler, H., & Brenner, H. (2006). The diagnosis of a smoking-related disease is a prominent trigger for smoking cessation in a retrospective cohort study. J Clin Epidemiol, 59(1), 82-89.

Vanderschuren, L. J., Di Ciano, P., & Everitt, B. J. (2005). Involvement of the dorsal striatum in cue-controlled cocaine seeking. J Neurosci, 25(38), 8665-8670.

Veeneman, M. M., Broekhoven, M. H., Damsteegt, R., & Vanderschuren, L. J. (2011). Distinct Contributions of Dopamine in the Dorsolateral Striatum and Nucleus Accumbens Shell to the Reinforcing Properties of Cocaine. Neuropsychopharmacology.

Volkow, N. D., Wang, G. J., Fowler, J. S., Logan, J., Gatley, S. J., MacGregor, R. R., et al. (1996). Measuring age-related changes in dopamine D2 receptors with 11C-raclopride and 18F-N-methylspiroperidol. Psychiatry Res, 67(1), 11-16.

Zhou, Y., Fathali, N., Lekic, T., Ostrowski, R. P., Chen, C., Martin, R. D., et al. (2011). Remote limb ischemic postconditioning protects against neonatal hypoxic-ischemic brain injury in rat pups by the opioid receptor/Akt pathway. Stroke, 42(2), 439-444.

Zubieta, J. K., Dannals, R. F., & Frost, J. J. (1999). Gender and age influences on human brain mu-opioid receptor binding measured by PET. Am J Psychiatry, 156(6), 842-848.

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Effects of Systemic Administration of Oxytocin on Contextual Fear Extinction in a Rat Model of Post-Traumatic Stress Disorder

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ABSTRACT

Introduction: One of the hallmark symptoms of posttraumatic stress disorder (PTSD) is the impaired extinction of traumatic memory. Single prolonged stress (SPS) has been suggested as an animal model of PTSD, since SPS rats exhibited the impaired fear extinction. Oxytocin (OXT) has been recently suggested as a potential pharmacotherapy for treatment of PTSD. In this study, using SPS rats we investigated the effects of multiple systemic administration of OXT on contextual fear extinction.

Methods: SPS was conducted in three stages: restraint for 2 h, forced swim for 20 min, and diethyl ether anesthesia, and then left undisturbed in their home cage for 7 days. In the SPS group, 7 days after SPS treatment, contextual fear conditioning was performed (on day 0), and then extinction training was performed on each of four consecutive days following fear conditioning. In the sham group, the procedures were similar except that SPS treatment was not performed.

Results: During extinction trial (10 min) freezing behavior was recorded. OXT (1, 10, 100 and 1000µg/kg) was administrated (I.P) immediately after each extinction trial. SPS rats exhibited significant impairment of contextual fear extinction as compared with sham rats. While there was no significant difference in the freezing levels between SPS and Sham rats 24 h after the fear conditioning, the freezing levels in SPS rats were significantly higher than those in sham rats after the second extinction training. Systemic OXT delayed fear extinction in sham rats as compared with sham-saline treated animals. No effect of OXT was found in SPS rats.

Discussion: These findings indicate that increasing OXT transmission during fear memory reactivation delays fear extinction, and thus, the recommendation of OXT for PTSD treatment should be considered with caution.

Key Words:
Oxytocin,
PTSD,
Fear Conditioning,
Fear Extinction.

1. Introduction

Posttraumatic stress disorder (PTSD) is a psychiatric disorder that develops following the experience of life-threatening traumatic events, characterized by symptoms such as persistent re-experiencing of the traumatic event, avoidance of stimuli associated with the trauma, numbing of general responsiveness, and increased arousal (Pitman 1997). The neuronal mechanisms underlying fear conditioning and impaired fear extinction were suggested to be involved in the production of re-experiencing symptoms, such as intrusive memory and, in PTSD (Charney, Deutch, Krystal, Southwick, & Davis 1993). Thus, extinction learning (a reduction in conditioned fear response when the conditioned stimulus is repeatedly presented in the absence of an unconditioned stimulus) plays an important role in the treatment of PTSD.

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In fact, cognitive behavioral therapy (CBT), the most commonly used approach for the treatment of PTSD, relies on extinction-based mechanisms (Rothbaum & Davis 2003). Numerous studies have demonstrated CBT as an effective treatment for PTSD (Agorastos, Marmar, & Otte 2011, Olatunji, Cisler, & Deacon 2010, Roberts, Kitchiner, Kenardy, & Bisson 2009). However, many patients fail to achieve remission with CBT (Cloitre 2009, Devilly & Huther 2008). Recent studies have suggested that adding some pharmacologic agents to CBT, particularly those involved in neural control of fear extinction, can augment treatment efficacy in PTSD patients (Davis, Ressler, Rothbaum, & Richardson 2006, Dunlop, Mansson, & Gerardi 2012).

Oxytocin (OXT), a nine-amino acid peptide, is synthesized in the hypothalamus and secreted into the blood stream in the neurohypophysis as a neuro-hormone (Gimpl & Fahrenholz 2001). As a hormone, it mainly regulates a variety of biological functions such as reproduction-associated processes (Ivell, Balvers, Rust, Bathgate, & Einspanier 1997), social recognition (Keverne & Curley 2004), maternal behavior (Pedersen, Ascher, Monroe, & Prange 1982), and neuroendocrine regulation of the stress response (Neumann 2002). In addition to endocrine functions, OXT acts as a neurotransmitter in a variety of brain structures including the septum, hippocampus, and central amygdala in response to various stressful stimuli (Bosch, Meddle, Beiderbeck, Douglas, & Neumann 2005, Neumann 2008). Exogenous OXT has anxiolytic effects in both animals and humans (Heinrichs & Domes 2008), inhibit the activity of the hypothalamic–pituitary–adrenal (HPA) axis (Neumann, Wigger, Torner, Holsboer, & Landgraf 2000), and reduces activation of the amygdala to threatening faces, thereby reducing the autonomic and behavioral manifestation of fear in healthy volunteers and patients with social anxiety disorder (Kirsch et al. 2005, Labuschagne et al. 2010). These findings suggest that OXT has anxiolytic and antistress effects in both humans and rodents. Since PTSD is marked by impairments in anxiety/stress regulation and hyperactivity of the amygdala (Rauch et al. 2000, Shin, Rauch, & Pitman 2006),, OXT might be a good candidate for the treatment of PTSD (Olff, Langeland, Witteveen, & Denys 2010, Viviani et al. 2011).

Single prolonged stress (SPS) has been proposed as a valid animal model of PTSD, since rats exposed to SPS illustrate enhanced negative feedback of the HPA axis (Kohda et al. 2007), a sustained exaggeration of the acoustic startle response, increased anxiety–like behavior in the elevated plus maze, and increased contextual fear (Yamamoto et al. 2009). These responses resemble the clinical symptoms observed in PTSD patients (Pitman 1997). In the present experiments, OXT was administered systemically at different doses to investigate its effects on the extinction of fear response in a rat model of PTSD.

2. Methods

2.1. Animals

Male Wistar rats (200-300g) were housed five per cage, maintained on a 12-h light/dark cycle (light on from 08:00 to 20:00), and fed and watered ad libitum. All procedures were conducted in agreement with the National Institutes of Health Guide for Care and Use of Laboratory Animals. All behavioral experiments were done between 9am to 2pm.

2.2. Drugs

OXT (Sigma-Aldrich, USA) was dissolved in saline and administrated interaperitoneally at doses of 1, 10, 100, or 1000 µg/2ml/kg. The choice of doses was based on pilot and previous studies (Kovács, Vécsei, & Telegdy 1978, Missig, Ayers, Schulkin, & Rosen 2010, Petersson, Hulting, & Uvnäs-Moberg 1999).

2.3. SPS Procedure

SPS is an animal model of PTSD that first proposed by Liberzon, Krstov and Young (1997). According to this method, SPS was conducted in three stages: restraint for 2 h, forced swim for 20 min, and diethyl ether anesthesia. Each rat was restrained for 2 h by placing it inside a disposable clear polyethylene cone bag with only the tail protruding. The wide end of the cone was closed with tape at the base of the tail. The bag size was adjusted according to the size of the rat in order to achieve complete immobilization. A hole in the narrow end of the cone allowed the rats to breathe freely. After immobilization, the rats were individually placed in a clear acrylic cylinder (240 mm D, 500 mm H), filled two-thirds from the bottom with water (24 °C), and forced to swim for 20 min. Following 15 min recuperation, they were exposed to diethyl ether (Sigma-USA) until loss of consciousness and then left undisturbed in their home cages for 1 week. Sham rats were left undistributed in their home cage prior to the contextual fear conditioning.

2.4. Contextual fear Conditioning and Extinction Training

An automated rodent fear conditioning system (TSE, Bad Homburg, Germany) was used to study contextual

fear conditioning of each rat. Contextual fear conditioning took place in a conditioning box. The walls and the ceiling of the box were constructed of clear Plexiglass. The floor of the box was made of 25 stainless steel rods (6 mm in diameter, 12 mm apart) through which footshock could be delivered from a constant current source. The box was enclosed in a sound attenuating chamber. The chamber was illuminated by a single house light, and was cleaned with 5% ethanol before and after utilization. Ventilation fans provided continuous background noise (68 dB) during the experiment. A software program was used to control the test in the box, and to collect, display and store all experimental data for "off-line" analysis.

General procedures for contextual fear conditioning have previously been described (Abrari, Rashidy-Pour, Semnanian, & Fathollahi 2008). Briefly, contextual fear conditioning took place in a conditioning box. On day 1, the rats were placed into the chamber and after 3 min received two footshocks at 30 s min intervals. Each shock was 1.5 mA and 4 s duration. Rats were left in the conditioning box for 30 s after termination of the procedure and returned to their home cage.

Extinction training was defined as the repetitive exposure to the contextual box in the absence of footshock. One-day after contextual training, rats were placed for 10 min in the same context and the percentage of time animal spent freezing (characterized by the absences of all visible movement expect respiration) was measured using automated procedures. Time threshold for freezing behavior was set on 3 second and. freezing was interpreted whenever the animal was not moving for more than this duration. In a similar way, extinction training was performed on each of four consecutive days following fear conditioning (Fig. 1)

2.5. Locomotor Activity Measurement

Locomotor activity of animal was measured using an automated activity monitor system (TSE infraMot, TSE, Bad Homburg, Germany) as previously described elsewhere (Rashidy-Pour, Sadeghi, Taherain, Vafaei, & Fathollahi, 2004). Spontaneous locomotor activity of each rat was measured for five 2-min intervals. Only one animal was placed in each activity chamber per measurement time.

2.6. Statistical Analysis

Data were expressed as a means ± SEM and were analyzed with analysis of variance (ANOVA) with repeated measure and the unpaired-Student t-test. All post hoc comparisons were made using Tukey's multiple comparison test. Values of $P < 0.05$ were considered significant.

2.7. Experiments

2.7.1. Effects of SPS on Spontaneous Motor Activity

In this experiment, we examined influence of SPS on spontaneous motor activity. Seven days after SPS, the lev-

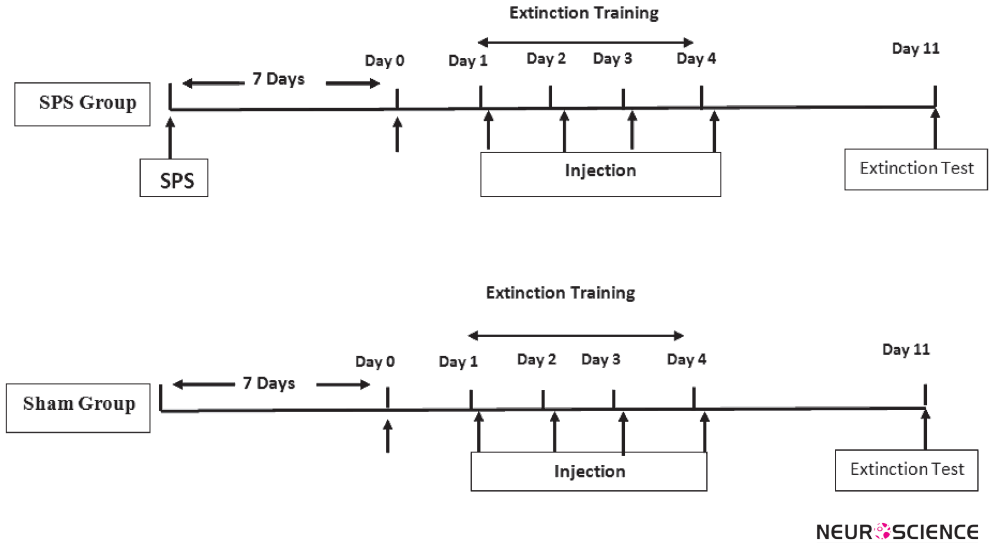

Figure 1. Experimental groups and treatment procedures. In the SPS group, 7 days after SPS treatment, contextual fear conditioning was performed (on day 0), and then extinction training was performed on each of four consecutive days following fear conditioning. In the sham group, the procedures were similar except that SPS treatment was not performed.

els of spontaneous motor activity of sham and SPS rats were measured according to procedures explained above.

2.7.2. Effects of SPS on Fear Extinction

In the first experiment, we investigated influence of SPS on fear extinction. Rats were randomly divided into two SPS and sham groups (n= 8-10 in each group). Sham group: animals of this group were left undistributed without handling in their home cages. SPS group: SPS procedure was performed on these animals according the procedures described above. Fear conditioning and extinction training were performed as described above. Immediately following each extinction trial, each rat received physiological saline. In addition, to examine the sustained effect of saline upon fear extinction, we evaluated the freezing responses of animals of each group one week after the fourth extinction training period (Fig. 1).

2.7.3. Effect of systemic OXT on Fear Condition.

Animals were randomly divided into 4 sham and 4 SPS groups. Fear conditioning and extinction training were performed as described above. Immediately following extinction, each group of rat was administrated 1, 10, 100, or 1000 µg/2ml/kg of OXT or saline (Fig. 2).

3. Results

3.1. SPS does not Impair Spontaneous Motor Activity

The locomotor activity results are shown in Fig. 2A. There were no significant differences between groups in total activity recorded for 10 min period (t_{12}= 0.25; P = 0.80). A two-way ANOVA (group × time) of locomotor activity at each two min interval of the 10 min test showed no significant effect of groups ($F_{1,12}$= 0.06; P = 0.80), a significant effect of time ($F_{4,56}$=6.66; P=0.0002), and no significant interaction between both factors ($F_{4,56}$=0.75; P=0.55). Post-hoc analysis indicated that in all groups locomotor activity was increased during the first two min interval ($P<0.05$ for all comparisons). These results indicate that SPS did not affect spontaneous locomotor activity.

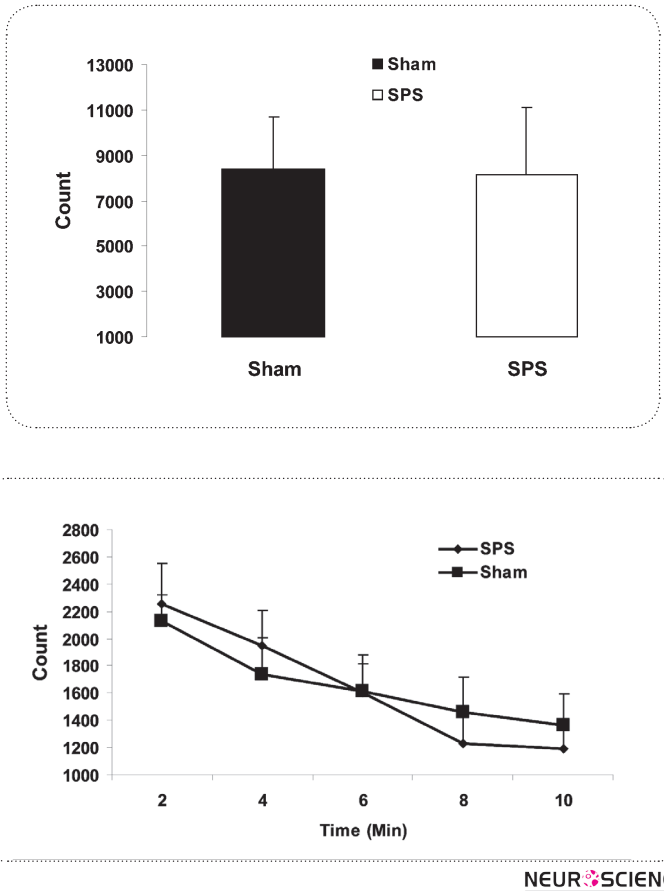

Figure 2. Effects of SPS procedures on spontaneous motor activity. (A) mean (±S.E.M.) total locomotor activity for a 10-min period; (B) mean (±S.E.M.) locomotor activity over the 10-min test (2-min interval).

3.2. SPS Impairs Contextual Fear Extinction

Fig. 3 shows freezing responses of Sham and SPS groups across the multiple extinction training. Two-way ANOVA with repeated measures on data from experiment 2 indicated a significant main effect of day (F4,64= 14.67, P=0.0001), stress (F 1,16= 5.77, P=0.029), and no significant interaction between day and stress (F(4,72)= 1.2,P=0.8). Post-hoc comparison using the unpaired Student's t-test indicated that the freezing levels of SPS and sham groups did not differ on day1, suggesting that CFC was successful in both groups. During extinction training, however, a significant difference in the freezing levels was found on days 4 (P<0.01), and 11 (P<0.001) between both groups (Fig. 3).

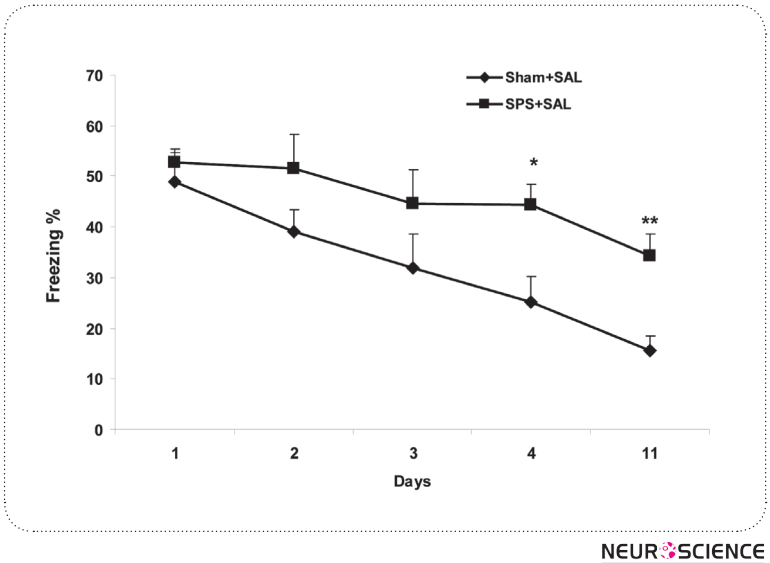

Figure 3. Effects of SPS on contextual fear extinction. Data are expressed as mean ± SEM. Results indicated that the freezing levels of SPS and sham groups did not differ on day1, suggesting that CFC was successful in both groups. During extinction training, however, a significant difference in the freezing levels was found on days 4, and 11 between both groups.

3.3. Effect of OXT on Fear Extinction in Sham and SPS Rats

A three-way ANOVA with repeated measure showed significant effects of groups (F1,348=9.731,P=0.002), of treatments (F4,348=7.89,P=0.0001) and of days (F3,348)=4.14,P=0.007). Post-hoc comparisons indicated that freezing levels of sham rats receiving doses of 10,100 or 1000 µg/kg of OXT were significantly higher than those rats receiving saline in day 2 to day 5 (all, P<0.05). No significant differences was found between saline and OXT-treated SPS rats.

4. Discussion

Using an animal model of PTSD, we investigated the effects of OXT on the impaired fear extinction in SPS and sham rats. While there was no significant difference in the freezing levels between SPS and sham rats 24 h after the fear conditioning, the freezing levels in SPS rats were significantly higher than those in sham rats after the forth, and fifth extinction training. Moreover, we found no differences in locomotor activity between SPS and sham groups. These findings rule out the possibility that the impaired extinction of SPS rats is due to disturbances of motor function.

Our findings are in agreement with previous studies indicating that SPS impairs contextual fear extinction, but does not disrupt sensory-motor function (Iwamoto, Morinobu, Takahashi, & Yamawaki 2007, Yamamoto et al. 2007). We found that repeated OXT administration slowed down the extinction of contextual fear in sham rats. In fact, sham rats receiving doses of 10,100 or 1000 µg/kg of OXT showed higher freezing levels in subsequent testes than sham rats receiving saline. This effect was also found 7 days after the fourth extinction training. However, in SPS rats, OXT had no impairing effect. One possible interpretation is that SPS rats have already displayed the impaired extinction and thus, the

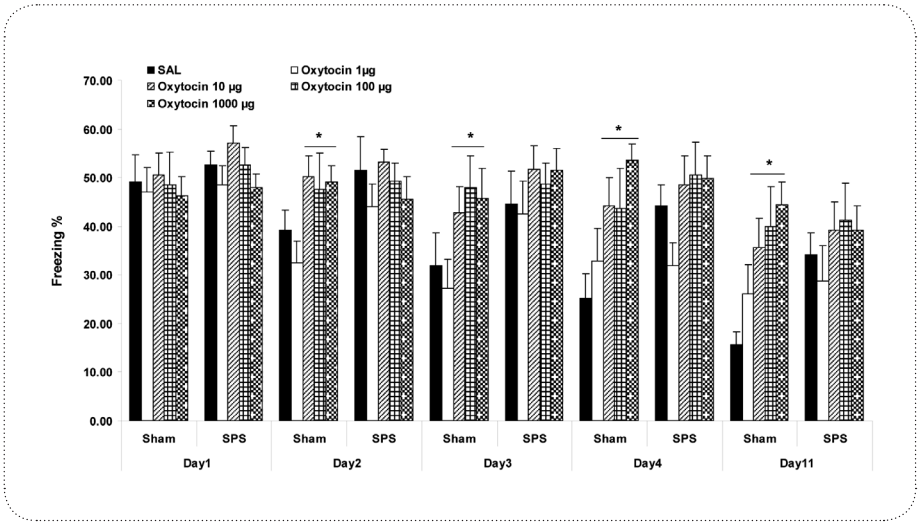

Figure 4. Effects of multiple injections of oxytocin on the extinction of fear response in sham and SPS rats. Data are expressed as mean ± SEM. *P<0.01, **P<0.001 as compared with saline-treated sham rats in the same day.

freezing level of SPS rats might reach a ceiling and this prevents us from detecting the impairing effect of oxytocin. Our findings are in agreement with a recent study showing that central administration of OXT impairs cued fear extinction in both rats and mice (Toth, Neumann, & Slattery, 2012). In that study, OXT was injected before extinction training, and thus, the drug influenced the acquisition of extinction. OXT administration before extinction training not only influences the acquisition of extinction, but also affects post-training consolidation process. We injected OXT immediately following each extinction training. In this time, the memory is active and the consolidation process is occurring. Thus, the injected OXT influenced the consolidation of extinction learning. The lower dose of OXT (1µg) was ineffective. The impaired extinction was not a result of reduction in general locomotor activity because OXT was injected following each extinction trial and the animals retested 24 h later. Given that the half-life of OXT is very short, during this interval the injected OXT is metabolized and eliminated from the plasma. Additionally, we found no differences in general locomotor activity between saline, OXT-treated, sham and SPS animals (data not shown).

Our findings are in contrast with previous studies showing that administration of OXT before extinction training facilitated fear extinction. For example, injection of OXT into the central amygdala before extinction training facilitated the fear extinction (Viviani et al. 2011, Wiesma, Sluyter, & Driscoll 1992). The central amygdala coordinates the behavioral and physiological correlates of fear expression (LeDoux, Iwata, Cicchetti, & Reis 1988). In this study, however, OXT was administered systemically, which is likely to explain the inconsistent results. Despite the fact that peripherally administered OXT may reach and influence the central amygdala, it may not do so in a concentration adequate to enhance fear extinction. Additionally, it is likely to influence brain areas which increase fear responses, such as the basolateral amygdala (BLA) and hippocampus. Both structures play an important role in fear modulation and have oxytocin receptors (Phillips & LeDoux 1992, Vaccari, Lolait, & Ostrowski 1998). Much more research is needed to determine the role of these structures in the impairing effects of oxytocin on fear extinction.

In general, peptides, including OXT, pass the blood-brain barrier with difficulty (Zaidi & Heller, 1974). Thus, central content of OXT should not be affected significantly by the alteration of peripheral peptide concentration following its systemic administration (Engelmann, Wotjak, Neumann, Ludwig, & Landgraf 1996). These findings suggest that intraperitoneally administered OXT may alter fear conditioning without directly influencing brain systems. A possible mechanism that may mediate the effects of OXT on fear extinction is the inhibition of glucocorticoid release from the adrenal glands by OXT (Petersson et al. 1999). Previous studies have shown that decreasing corticosterone levels before extinction training by icvntracerebroventricular and BLA administration of metyrapone, a blocker of glucocorticoid synthesis blocks fear extinction (Barrett

& Gonzalez-Lima 2004, Jin, Lu, Yang, Ma, & Li 2007, Yang, Chao, Ro, Wo, & Lu 2006). Conversely, injections of glucocorticoid receptor agonists before extinction training facilitate fear extinction training (Yang, Chao, & Lu 2005, Yang et al. 2006)

In conclusion, our study shows that increasing OXT transmission at time of memory reactivation delays the extinction of conditioned fear response, and thus, much attention is required before commencing OXT for treatment of PTSD. Future research is required to determine the underlying mechanisms of oxytocin inhibitory effects on fear extinction. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Abrari, K., Rashidy-Pour, A., Semnanian, S. & Fathollahi, Y. (2008) Administration of corticosterone after memory reactivation disrupts subsequent retrieval of a contextual conditioned fear memory: dependence upon training intensity. Neurobiology of Learning and Memory 89, 178-184.

Agorastos, A., Marmar, C. R. & Otte, C. (2011) Immediate and early behavioral interventions for the prevention of acute and posttraumatic stress disorder. Current Opinion in Psychiatry 24, 526-532.

Barrett, D. & Gonzalez-Lima, F. (2004) Behavioral effects of metyrapone on Pavlovian extinction. Neuroscience Letters 371, 91-96.

Bosch, O. J., Meddle, S. L., Beiderbeck, D. I., Douglas, A. J. & Neumann, I. D. (2005) Brain oxytocin correlates with maternal aggression: link to anxiety. The Journal of Neuroscience 25, 6807-6815.

Charney, D. S., Deutch, A. Y., Krystal, J. H., Southwick, S. M. & Davis, M. (1993) Psychobiologic mechanisms of posttraumatic stress disorder. Archives of General Psychiatry 50, 294.

Cloitre, M. (2009) Effective psychotherapies for posttraumatic stress disorder: a review and critique. CNS Spectr 14(1 Suppl 1), 32-43.

Davis, M., Ressler, K., Rothbaum, B. O. & Richardson, R. (2006) Effects of D-cycloserine on extinction: translation from preclinical to clinical work. Biological Psychiatry 60, 369-375.

Devilly, G. J. & Huther, A. (2008) Perceived distress and endorsement for cognitive-or exposure-based treatments following trauma. Australian Psychologist 43, 7-14.

Dunlop, B.W, Mansson, E. & Gerardi, M. (2012) Pharmacological Innovations for Posttraumatic Stress Disorder and Medication-Enhanced Psychotherapy. Current Pharmaceutical Design 18, 5645-5658.

Engelmann, M., Wotjak, C.T., Neumann, I., Ludwig, M. & Landgraf, R. (1996) Behavioral consequences of intracerebral vasopressin and oxytocin: focus on learning and memory. Neuroscience and Biobehavioral Review 20,341-58.

Gimpl, G. & Fahrenholz, F. (2001) The oxytocin receptor system: structure, function, and regulation. Physiological Reviews 81, 629-683.

Heinrichs, M. & Domes, G. (2008) Neuropeptides and social behaviour: effects of oxytocin and vasopressin in humans. Progress in Brain Research 170, 337-350.

Ivell, R., Balvers, M., Rust, W., Bathgate, R. & Einspanier, A. (1997) Oxytocin and male reproductive function. In: The Fate of the Male Germ Cell, ed.^eds. Springer.

Iwamoto, Y., Morinobu, S., Takahashi, T. & Yamawaki, S. (2007) Single prolonged stress increases contextual freezing and the expression of glycine transporter 1 and vesicle-associated membrane protein 2 mRNA in the hippocampus of rats. Progress in Neuro-Psychopharmacology and Biological Psychiatry 31, 642-651.

Jin, X. C., Lu, Y. F., Yang, X. F., Ma, L. & Li, B. M. (2007) Glucocorticoid receptors in the basolateral nucleus of amygdala are required for postreactivation reconsolidation of auditory fear memory. European Journal of Neuroscience 25, 3702-3712.

Keverne, E. B. & Curley, J. P. (2004) Vasopressin, oxytocin and social behaviour. Current Opinion in Neurobiology 14, 777-783.

Kirsch, P., Esslinger, C., Chen, Q., Mier, D., Lis, S., Siddhanti, S., Gruppe, H., Mattay, V. S., Gallhofer, B. & Meyer-Lindenberg, A. (2005) Oxytocin modulates neural circuitry for social cognition and fear in humans. The Journal of neuroscience 25, 11489-11493.

Kohda, K., Harada, K., Kato, K., Hoshino, A., Motohashi, J., Yamaji, T., Morinobu, S., Matsuoka, N. & Kato, N. (2007) Glucocorticoid receptor activation is involved in producing abnormal phenotypes of single-prolonged stress rats: a putative post-traumatic stress disorder model. Neuroscience 148, 22-33.

Kovács, G. L., Vécsei, L. & Telegdy, G. (1978) Opposite action of oxytocin to vasopressin in passive avoidance behavior in rats. Physiology & Behavior 20, 801-802.

Labuschagne, I., Phan, K. L., Wood, A., Angstadt, M., Chua, P., Heinrichs, M., Stout, J. C. & Nathan, P. J. (2010) Oxytocin attenuates amygdala reactivity to fear in generalized social anxiety disorder. Neuropsychopharmacology 35, 2403-2413.

LeDoux, J. E., Iwata, J., Cicchetti, P. & Reis, D. (1988) Different projections of the central amygdaloid nucleus mediate

autonomic and behavioral correlates of conditioned fear. The Journal of Neuroscience 8, 2517-2529.

Liberzon, I., Krstov, M. & Young, E. A. (1997) Stress-restress: effects on ACTH and fast feedback. Psychoneuroendocrinology 22, 443-453.

Missig, G., Ayers, L. W., Schulkin, J. & Rosen, J. B. (2010) Oxytocin reduces background anxiety in a fear-potentiated startle paradigm. Neuropsychopharmacology 35, 2607-2616.

Neumann, I. (2008) Brain oxytocin: a key regulator of emotional and social behaviours in both females and males. Journal of Neuroendocrinology 20, 858-865.

Neumann, I., Wigger, A., Torner, L., Holsboer, F. & Landgraf, R. (2000) Brain oxytocin inhibits basal and stress-induced activity of the hypothalamo-pituitary-adrenal axis in male and female rats: partial action within the paraventricular nucleus. Journal of Neuroendocrinology 12, 235-244.

Neumann, I. D. (2002) Involvement of the brain oxytocin system in stress coping: interactions with the hypothalamo-pituitary-adrenal axis. Progress in Brain Research 139, 147-162.

Olatunji, B. O., Cisler, J. M. & Deacon, B. J. (2010) Efficacy of cognitive behavioral therapy for anxiety disorders: a review of meta-analytic findings. Psychiatric Clinics of North America 33, 557.

Olff, M., Langeland, W., Witteveen, A. & Denys, D. (2010) A psychobiological rationale for oxytocin in the treatment of posttraumatic stress disorder. CNS Spectr 15, 522-530.

Pedersen, C. A., Ascher, J. A., Monroe, Y. L. & Prange, A. J. (1982) Oxytocin induces maternal behavior in virgin female rats. Science 216, 648-650.

Petersson, M., Hulting, A.-L. & Uvnäs-Moberg, K. (1999) Oxytocin causes a sustained decrease in plasma levels of corticosterone in rats. Neuroscience letters 264, 41-44.

Phillips, R. & LeDoux, J. (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behavioural Neuroscencei 106, 274-285.

Pitman, R. K. (1997) Overview of biological themes in PTSD. Annals of the New York Academy of Sciences 821, 1-9.

Rashidy-Pour, A., Sadeghi, H., Taherain, A.A., Vafaei, A.A. & Fathollahi Y (2004) The effects of acute restraint stress and dexamethasone on retrieval of long-term memory in rats: an interaction with opiate system. Behavioral Brain Research. 154,193-8.

Rauch, S. L., Whalen, P. J., Shin, L. M., McInerney, S. C., Macklin, M. L., Lasko, N. B., Orr, S. P. & Pitman, R. K. (2000) Exaggerated amygdala response to masked facial stimuli in posttraumatic stress disorder: a functional MRI study. Biological Psychiatry 47, 769-776.

Roberts, N., Kitchiner, N., Kenardy, J. & Bisson, J. (2009) Multiple session early psychological interventions for the prevention of post-traumatic stress disorder. Cochrane Database of Systematic Reviews 3.

Rothbaum, B. O. & Davis, M. (2003) Applying learning principles to the treatment of Post-Trauma reactions. Annals of the New York Academy of Sciences 1008, 112-121.

Shin, L. M., Rauch, S. L. & Pitman, R. K. (2006) Amygdala, medial prefrontal cortex, and hippocampal function in PTSD. Annals of the New York Academy of Sciences 1071, 67-79.

Toth I, Neumann ID, & Slattery DA(2012) Central administration of oxytocin receptor ligands affects cued fear extinction in rats and mice in a timepoint-dependent manner. Psychopharmacology (Berl). 223,149-58.

Vaccari, C., Lolait, S. J. & Ostrowski, N. L. (1998) Comparative distribution of vasopressin V1b and oxytocin receptor messenger ribonucleic acids in brain. Endocrinology 139, 5015-5033.

Viviani, D., Charlet, A., van den Burg, E., Robinet, C., Hurni, N., Abatis, M., Magara, F. & Stoop, R. (2011) Oxytocin selectively gates fear responses through distinct outputs from the central amygdala. Science Signaling 333, 104.

Wiersma, A., Sluyter, S. & Driscoll, P. (1992) Opposite effects of central amygdaloid vasopressin and oxytocin on the regulation of conditioned stress responses in male rats. Oxytocin in Maternal, Sexual, and Social Behaviors 460.

Yamamoto, S., Morinobu, S., Fuchikami, M., Kurata, A., Kozuru, T. & Yamawaki, S. (2007) Effects of single prolonged stress and D-cycloserine on contextual fear extinction and hippocampal NMDA receptor expression in a rat model of PTSD. Neuropsychopharmacology 33, 2108-2116.

Yamamoto, S., Morinobu, S., Takei, S., Fuchikami, M., Matsuki, A., Yamawaki, S. & Liberzon, I. (2009) Single prolonged stress: toward an animal model of posttraumatic stress disorder. Depression and Anxiety 26, 1110-1117.

Yang, Y.-L., Chao, P.-K. & Lu, K.-T. (2005) Systemic and intra-amygdala administration of glucocorticoid agonist and antagonist modulate extinction of conditioned fear. Neuropsychopharmacology 31, 912-924.

Yang, Y.-L., Chao, P.-K., Ro, L.-S., Wo, Y.-Y. P. & Lu, K.-T. (2006) Glutamate NMDA receptors within the amygdala participate in the modulatory effect of glucocorticoids on extinction of conditioned fear in rats. Neuropsychopharmacology 32, 1042-1051.

Zaidi, S,M. & Heller, H. (1974) Can neurohypophysial hormones cross the blood-cerebrospinal fluid barrier? J Endocrinol. 60,195-6.

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Use of Colchicine in Cortical Area 1 of the Hippocampus Impairs Transmission of Non-Motivational Information by the Pyramidal Cells

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ABSTRACT

Colchicine, a potent neurotoxin derived from plants, has been recently introduced as a degenerative toxin of small pyramidal cells in the cortical area 1 of the hippocampus (CA1). In this study, the effect of the alkaloid in CA1 on the behaviors in the conditioning task was measured. Injections of colchicine (1,5 µg/rat, intra-CA1) was performed in the male Wistar rats, while the animals were settled and cannulated in a stereotaxic apparatus. In the control group solely injection of saline (1 µl/rat, intra-CA1) was used. One week later, all the animals passed the saline conditioning task using a three-day schedule of an unbiased paradigm. They were administered saline (1 ml/kg, s.c.) twice a day throughout the conditioning phase. To evaluate the possible effects of cell injury by the toxin on the pyramidal cells, both the motivational signals while in the conditioning box and the non-motivational locomotive signs of the treated and control rats were measured. Based on the present study the alkaloid caused no change in the score of place conditioning, but affected both the sniffing and grooming behaviors in the group that received colchicine. However, the alkaloid did not show the significant effect on the rearing or compartment entering in the rats. According to the findings, the intra-CA1 injection of colchicine may impair the neuronal transmission of non-motivational information by the pyramidal cells in the dorsal hippocampus.

1. Introduction

Traditional lesion-producing methods by injecting toxic analogs of presumed neurotransmitters have received condemnation due to the lack of lesion selectivity in specific cell populations. This fact causes a serious problem whenever one attempts to ascribe the effects on the neurophysiology and neuronal transmission to the removal of a specific cell population. Thus, considerable interest has been generated in recent years by drugs that show a selective neurotoxic effect between cell populations.

Colchicine is a plant alkaloid (Alali, et al., 2005), which is known as a potent inhibitor of physiological processes. The alkaloid specifically binds to the receptor site of tubulin (Lockwood, 1979) and blocks mitosis (Mundy and Tilson, 1990).

Goldschmidt and Steward (1980) have previously demonstrated colchicine as a substance that preferentially destructs granulocytes in the dentate gyrus. Since Correia and Lobert (2001), we believe that the colchicine toxin affects other cellular processes rather tubulin subunits. Similar findings have been provided by Porkhodadad et al (2011). In this experiment, the possible damage to pyramidal cells in the cortical area 1 (CA1) of the hippocampus was evaluated by the saline conditioning task.

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This research plan is important because neuronal functions are dependent on an intact cytoskeleton and cytoskeletal alterations are thought to be associated with neurological disorders (Selkoe, 1999). This phenomenon is common in cases of neurodegenerative pathology (Uchida, et al., 2004). Neural lesions also may impair learning and spatial memory (McNaughton, et al., 1989), because sub-regions of the hippocampus seem to have a role in the function of spatial and working memory processes (Okada and Okaichi, 2009).

2. Experimental Procedure

2.1. Animals

The animals were male Wistar rats (Pasteur Institute of Iran, Tehran, Iran) weighing between 250-350g at the start of the experiments. Animals were housed in standard cages in a controlled colony room (temperature 21±3 °C). They were maintained under a 12:12 h light/dark cycle with water and food provided ad libitum. Six to eight animals were used in each experiment and all experiments were approved by the local ethics committee at Shahed University (Document No: 282/Nov 21, 2012).

2.2. Drugs

The drug colchicine (Merck Co., Germany) used in the present study was prepared freshly in sterile 0.9% NaCl solution. A mixture level of 5:2 of ketamine (100 mg/kg) and xylazine (20 mg/kg) purchased from the Veterinary Organization of Iran were intraperitoneally injected to anesthetize the experiment animals. Vehicle injections were of the appropriate concentration of 0.9% physiological saline.

2.3. Surgery and Colchicine Injection

The animals were anesthetized and placed in a stereotaxic apparatus, with the incisor bar set at approximately 3.3 mm below horizontal zero to achieve a flat skull position. An incision was made to expose the rat's skull. Two holes were drilled in the skull at stereotaxic coordinates: AP-3.8 mm posterior to bregma, and L±1.8 to ±2.2 mm according to the atlas of Paxinos and Watson (2005). Two 21-gauge guide cannulae were inserted into the holes. In the animals receiving bilateral injections of colchicine into the cortical area 1 (CA1) of the hippocampus, the guide cannulae were lowered 2 mm below bregma through the holes drilled at the above-mentioned coordinates.

The colchicine (1,5 µg/rat) was administered intra-CA1 of the hippocampus while the cannulated rats were still immobilized by the stereotaxic apparatus. The guide cannulae were anchored with a jeweler's screw, and the incision was closed with dental cement. After that, the injection cannula that extended 1 mm beyond the guide cannulae was inserted into the guide cannula, through which the alkaloid (1,5 µg/rat) was gently injected into the site. The injection cannula was then left in place for another 60 seconds to facilitate the diffusion of the drug. One week after recovery, the rats passed the saline place conditioning using an unbiased procedure.

2.4. Place Conditioning Apparatus and Saline Conditioning Task

A two-compartment conditioned place preference apparatus (30x60x30 cm) was used in the experiments. Place conditioning was conducted using an unbiased procedure, of which the design and the apparatus were previously described (Karami, et al., 2002).

2.5. Place Conditioning Paradigm

The experiment consisted of the three following phases:

Pre-conditioning (Familiarization): On day 1 (before the conditioning phase), animals received one habituation session. They were placed in the middle line of the apparatus to move freely in the entire apparatus for 10 minutes. In this phase, the removable wall was raised 12cm above the floor. The time spent by rats in each compartment was recorded by an Ethovision system (model LVC-DV323ec of an Auto iris LG) located 120 cm above the apparatus. The behavior recorded by the system was then analyzed by an observer who had no knowledge of the treatments. None of the groups displayed a significant preference for one of the compartments, confirming that this procedure is unbiased.

Conditioning: This phase was started one day after the familiarization. The conditioning phase consisted of 6 saline pairings; the animals were simply injected saline (1 ml/kg, s.c.) twice a day with a 6 h interval. Saline administration in the conditioning phase was carried out during the light phase of a 12 h light/dark cycle (e.g. at 09.00 am and at 15.00 pm). This protocol was performed similarly in the control group (1 µl/rat saline given intra-CA1) as well as in the experimental groups (1,5 µg/rat colchicine given intra-CA1).

Testing (Post-conditioning): Test sessions were carried out on day 5, one day after the last conditioning session, in an injection saline-free state. Each animal was tested only once. For testing, the removable wall was raised 12 cm above the floor and each animal was allowed free ac-

cess to both compartments of the apparatus for 10 min. The time spent in the compartments on the day of testing was subtracted from the time measured during the familiarization phase and the testing phases and the result was expressed as mean ± S.E.M. The count of behavioral signs as expressed by the change in the number of signs per10 min in the apparatus was also measured.

2.6. Histological Verification

After completion of the experiments, the animals were decapitated after an overdose of chloroform. The brains were removed and fixed in a 10% formalin solution for 48 h before sectioning. Sections were taken through the brain areas of the cannulae placements, and the cannulae placements were verified using the atlas of Paxinos and Watson (2005). Data from rats with injection sites located outside the CA1 area were excluded from the analysis.

2.7. Cresyl Violet Stain

Thin (10-15 µm) brain sections provided by paraffin blocks were placed in 50%, 75%, and 95% ethanol for 1 min each and in 100% ethanol for 15 min. The sections were then rehydrated in 95%, 75%, and 50% ethanol and water for 1 min each and administered with cresyl violet solution (Merck Co., Germany) for 2 min. Subsequently, the sections were placed in 0.1% acetic acid in 75% ethanol for 30 sec, rinsed in water, dehydrated, and mounted in Entellan (Merck Co., Germany).

2.8. Statistical Analysis

The behavioral data were analyzed with one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison tests. P-values under 0.05 were considered significant. To analyze the histological data, the stained brain slices of the animals were examined by the Image Tool program (UTHSCSA ImageTool, version 2.03). After spatial and density calibrations with the intact (only anesthetized) samples, we made comparisons between the treated rats' brain sections and the calibrated samples to provide the data ± SEM at the significant levels.

3. Results

3.1. Cell Damage Induced by Colchicine in the Area of CA1 of Wistar Rats

The brain sections were analyzed with a qualitative tool (Image tool program) and showed a significant difference ($p<0.05$). The colchicine (1,5 µg/rat) was effective in inducing of lesion in the CA1 layer (Fig. 1) when compared with the intact (only anesthetized) one.

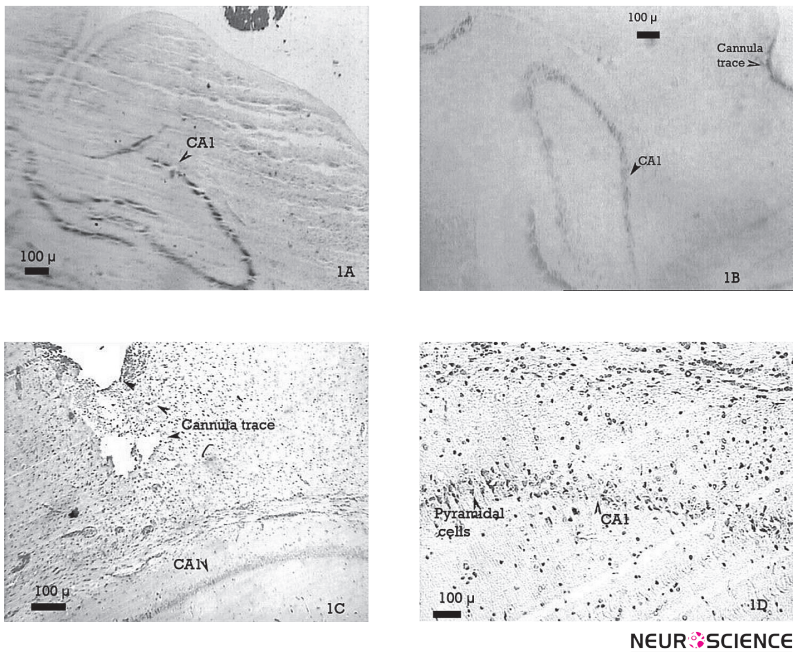

Figure 1. The histological verifications of the effect of colchicine lesion (1,5 µg/rat, intra-CA1) in Wistar rats. The injection of colchicine was done only once. After receiving the drug each animal was allowed to recover. The animal then passed the behavioral tests. The rats' brains were then collected in formalin to allow for histological confirmation. Based on the Image Tool program the damaging effect was clear in the rats' brains that received colchicine (Fig. 1B-1D, from small to large magnification). As shown in figured 1B-1D, colchicine was effective to induce cell damage in contrast to the intact (anesthetized) sample (Fig. 1A).

3.2. Effect of injection of Colchicine Intra-CA1 on Saline Conditioning

Bilateral injection of colchicine (1,5 µg/rat, intra-CA1), a plant derived alkaloid, before (1 week) starting the process of saline place conditioning, showed no significant difference in conditioning score of the the rats compared to the control group (p> 0.05) (Fig. 2).

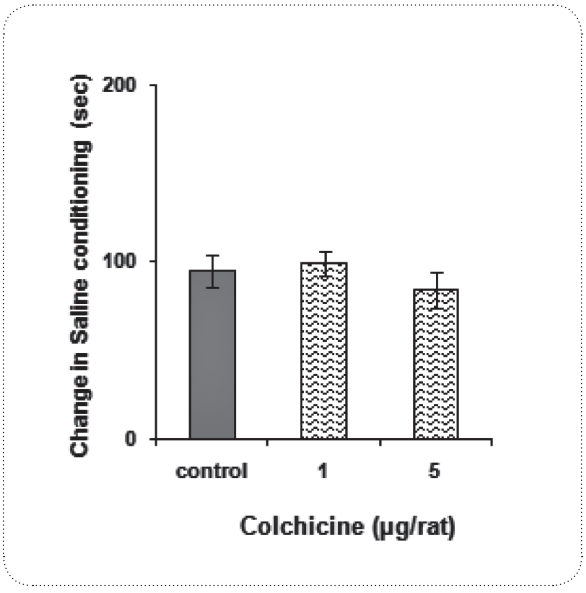

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Figure 2. Response to colchicine or saline (control), intra-CA1, in the saline place conditioning task. Each animal, after having recovered, was injected with saline (1 ml/kg, s.c.) twice per day throughout the conditioning phase. The animals were tested in the injection-free state on the day of testing to evaluate a simple type of learning notifying a link between the environment and the saline. Data are expressed as the score of changes in place preference and expressed as mean ±S.E.M. No difference between the drug-administered group and the vehicle was observed.

3.3. Effect of Injection of Colchicine Intra-CA1 on Behavioral Signs

Fig. 3 shows the effect of colchicine injection (1,5 µg/rat, intra-CA1) on behavioral signs in Wistar rats. Administration of the neurotoxin showed a significant effect on sniffing ($F_{2,15}=23.414$; $p<0.0001$) and grooming ($F_{2,15}=2.825$; $p<0.05$). However, no significant effect on wet dog shaking (WDS) or rearing was recorded.

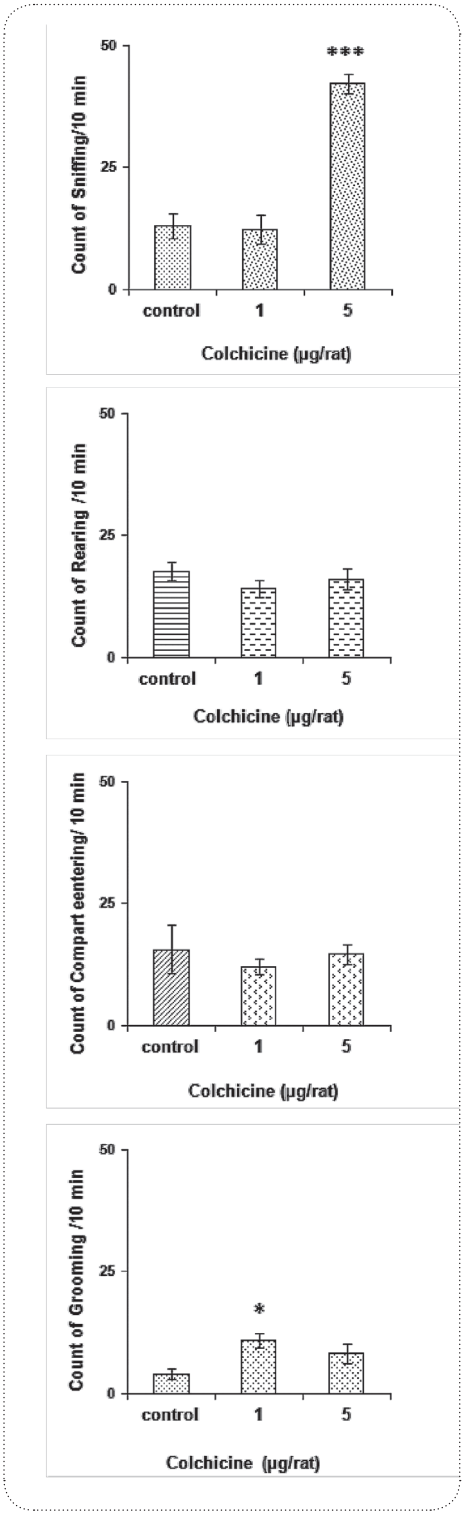

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Figure 3. The behavioral signs in male Wistar rats that received colchicine or saline (control) intra-CA1. The colchicine (1,5 µg/rat) or saline (1 ml/kg, µl/rat) was given one week before starting the unbiased conditioning paradigm. The animals were then injected saline (1 ml/kg, s.c.), twice daily for 3 days. Data are expressed as counts of behavioral signs per 10 min ±SEM. Post hoc analysis by Tukey showed the differences (*p< 0.05, ***p< 0.001) to the control.

4. Discussion

This research was designed to survey the effect of a potent plant neurotoxin, colchicine, on small pyramidal cells in the cortical area 1 (CA1) of the hippocampus and on the behavior in a conditioning task as well. According to this study the toxin showed degenerative effects on the pyramidal cells and produced a significant change in sniffing and grooming.

Colchicine is an alkaloid which is extracted from *Colchicum autumnale* L. This toxin binds to tubulin dimers which results in the formation of a tubulin–colchicine complex that acts primarily to prevent microtubule assembly (Panda, et al., 1995). In animal cells colchicine is usually considered as a lethal agent even at the lowest concentrations (Eigsti and Dustin, 1995; Rieder and Palazzo, 1992).

This work showed a damaging effect of colchicine on the small pyramidal cell population in the rat CA1 area. Supporting finding provides that the toxin colchicine irreversibly damages dendrites by disrupting their microtubular supporting network (Giuditta, et al., 2008), an effect which is caused by the selective toxic effect of colchicine on dentate granule cells (Walsh, et al., 1986). Furthermore, some other studies using doses of colchicine similar to those that were used in the present study have reported only reversible effects (Gajate, et al., 2000). To assess the neurotoxic effect of colchicine in the CA1 on learning programs, sniffing, rearing, grooming and compartment entering were analyzed for all rats within the saline conditioning task and the results provided some significant effects.

After place conditioning testing the experiment animals, those suffering from the lesion made by colchicine (1,5 µg/rat) in the CA1 area, exhibited different types of sniffing and grooming behaviors in comparison to the control saline (intra-CA1) treated group. It is proposed that this alkaloid inhibits the rapid axonal transport (Hanson and Edstrom, 1978) and produces long-lasting morphological changes in neurons and glia (Csillik, et al., 1977). It has also been suggested that the axonal membrane (Dziegielewska, et al., 1976) as well as dendrites (Partida-Sanchez, et al., 2000) are the site of action of colchicine, because these sites are rich in microtubules and contain specific receptors (Kumar, et al., 2009). A further important characteristic is that the CA1 neurons are responsible for simple learning processes like conditioning. In order to make clear responses to conditioned stimuli its neurons need complete entity and safe existence (Arushanyan and Beier, 2008). Thus, the neuronal responses are changed and exhibited a different type of sniffing and grooming in regard to the non-treated ones. Damage to CA1 pyramidal cells causes deficits in aspects of learning as present data show. It should be noted that the degree of learning impairment has been investigated by other researchers (Dillon, et al., 2008) and is proportional to the extent of damage within the cortical area. So, the colchicine treated groups were impaired in information processing such as odor sensing. This may underlie fine properties of the CA1 area.

Odor sampling by sniffing behavior in this paradigm has raised the idea that sniffing plays a critical role in odor information processing by shaping spatial and temporal patterns of afferent input to the olfactory bulb and through the patterns of higher level neural activity as well (Wesson, et al., 2008). Also, in a survey of the novelty task in rats with the CA1 lesions, an impairment in place conditioning was recorded (Okada and Okaichi, 2009; Zheng, et al., 2004).

Thus, the effects reported in the present study may be the consequence of an impaired neuronal transmission in pyramidal cells of the hippocampus, a process which consequently may be related to the observed data. Based on the findings in this study the small pyramidal cells can be pointed out as another target for the action of colchicine, while the investigators have suggested granular cells as the main candidate for destruction by the alkaloid.

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References

Alali, F. Q., El-Elimat, T., Li, C., Qandil, A., Alkofahi, A., Tawaha, K., Burgess, J.P., Yuka, N., Kroll, D. J., Navarro, H. A., Falkinham, J. O., Wani, M. C., Oberlies, N. H. (2005). New Colchicinoids from a Native Jordanian Meadow Saffron, *Colchicum brachyphyllum*: Isolation of the First Naturally Occurring Dextrorotatory Colchicinoid. Journal of Natural Products, 68, 173-178.

Arushanyan, É. B., Beier, É. V. (2008). The Hippocampus and Cognitive Impairments. Neuroscience and Behavioral Physiology, 38, 72-77.

Correia, J. J., Lobert, S. (2001). Physiochemical Aspects of Tubulin-interacting Antimitotic Drugs. Current Pharmaceutical Design, 7, 1213-1228.

Csillik, B., Knyihár, E., Elshiekh, A. A. (1977). Degenerative atrophy of central terminals of primary sensory neurons induced by blockade of axoplasmic transport in peripheral nerves. Experientia, 15, 33-35.

Dillon, G. M., Qu, X., Marcus, J. N., Dodart, J. C. (2008). Excitotoxic lesions restricted to the dorsal CA1 field of the hippocampus impair spatial memory and extinction learning in C57BL/6 mice. Neurobiology of Learning and Memory 90 (2008) 426–433

Dziegielewska, K. M., Saunders, N. R., Evans, C. A., Skacel, P. O., Häggendal, C. J., Heiwall, P. O., Dahalström, A. B. (1976). Effects of colchicine and vinblastine on axonal transport of choline acetyltransferase in rat sciatic nerve. Acta Physiologica Scandinavica, 96, 486-94.

Eigsti, O. J., Dustin, P. (1955). Colchicine in agriculture, medicine, biology and chemistry. The Ames, Iowa: Iowa State College Press.

Gajate, C., Barasoain, I., Andreu, J. M., Mollinedo, F. (2000). Induction of Apoptosis in Leukemic Cells by the Reversible Microtubule-disrupting Agent 2-Methoxy-5-(2',3',4'-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one: Protection by Bcl-2 and Bcl-XL and Cell Cycle Arrest. Cancer Research, 60, 2651-2659.

Giuditta, A., Chun, J. T., Eyman, M., Cefaliello, C., Bruno, A. P., Crispino, M. (2008). Local Gene Expression in Axons and Nerve Endings: The Glia-Neuron Unit. Physiological Reviews, 88, 515-555.

Goldschmidt, R. B., Steward, O. (1980). Preferential neurotoxicity of colchicine for granule cells of the dentate gyrus of the adult rat (selective toxicity/neuronal degeneration). Proceedings of the National Academy of Sciences of the United States of America, 77, 3047-3051.

Hanson, M., Edstrom, A. (1978). Mitosis inhibitors and axonal transport. International Review of Cytology. Supplement, 7, 373-402.

Karami, M., Zarrindast, M. R., Sepehri, H., Sahraei, H. (2002). Role of nitric oxide in the rat hippocampal CA1 area on morphine-induced conditioned place preference. European Journal of Pharmacology, 449, 113-119.

Kumar, A., Dogra, S., Prakash, A. (2009). Neuroprotective Effects of Centella asiatica against Intracerebroventricular Colchicine-Induced Cognitive Impairment and Oxidative Stress. International Journal of Alzheimer's Disease, doi:10.4061/2009/972178.

Laguzzi, R. F., Adrien, J., Bourgoin, S., Hamon, M. (1979). Effects of intraventricular injection of 6-hydroxydopamine in the developing kitten. I. On the sleep waking cycles. Brain Research, 160, 445– 459.

Lockwood, A. H. (1979). Molecules in mammalian brain that interact with the colchicines site on tubulin. Proceedings of the National Academy of Sciences of the United States of America, 76, 1184-1188.

McNaughton, B. L., Barnes, C. A., Meltzer, J., Sutherland, R. J. (1989). Hipbipotential mediator of neuronal death, survival and regeneration; hippocampal granule cells are necessary for normal spatial learning. Experimental Brain Research, 76, 485-496.

Mundy, W. R., Tilson, H. A. (1990). Neurotoxic effects of colchicine. Neurotoxicology, 11, 539-547.

Okada, k., Okaichi, H. (2009). Functional differentiation and cooperation among the hippocampal subregions in rats to effect spatial memory processes. Behavioral Brain Research, 181- 191.

Panda, D., Goode, B. L., Feinstein, S. C., Wilson, L. (1995). Kinetic stabilization of microtubule dynamics at steady state by tau and microtubule-binding domains of tau. Biochemistry (Washington), 34, 11117-11127.

Partida-Sanchez, S., Garibay-Escobar, A., Frixione, E., Parkhouse, R. M., Santos-Argumedo, L. (2000). CD45R, CD44 and MHC class II are signaling molecules for the cytoskeleton-dependent induction of dendrites and motility in activated B cells. European Journal of Immunology, 30, 2722–2728.

Paxinos, G., Watson, C. (2005). The rat brain in stereotaxic coordinates (2nd ed). Ontario: Academic press.

Rieder, C. L., Palazzo, R. E. (1992). Colcemid and the mitotic cycle. Journal of Cell Science, 102, 387-392.

Porkhodadad, S., Karami, M., Jalali Nadoushan, M. R. (2011). Positive effect of nitric oxide on morphine-induced place conditioning in Wistar rats treated by colchicine intra-hippocampal CA1. Journal of Clinical Toxicology, 1:1, doi. org/10.4172/2161-0494.1000102.

Selkoe, D. J. (1999). Translating cell biology into therapeutic advances in Alzheimer's disease. Nature, 399, A23–A31.

Uchida, K., Samejima, M., Okabe, A. (2004). Neuroprotective effects of melatonin against anoxia/aglycemia stress, as assessed by synaptic potentials and superoxide production in rat hippocampal slices. Journal of Pineal Research, 37, 215–222.

Walsh, T. J., Schulz, D. W., Tilson, H. A., Schmechel, D. E. (1986). Colchicine-induced granule cell loss in rat hippocampus: selective behavioral and histological alterations. Brain Research, 98, 23-36.

Wesson, D. W., Donahou, T. N., Johnson, M., Wachowiak, M. (2008). Sniffing behavior of mice during performance in odor-guided tasks. Chemical Senses, 33, 581–596.

Zheng,X. G., Tan, B. P., Luo, X. J., Xu, W., Yang, X. Y., Sui, N. (2004). Novelty-seeking behavior and stress-induced locomotion in rats of juvenile period differentially related to morphine place conditioning in their adulthood. Behavioral Processes, 65, 15–23.

Ecstasy-Induced Caspase Expression Alters Following Ginger Treatment

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ABSTRACT

Introduction: Exposure to 3-4, methylenedioxymethamphetamine (MDMA) leads to cell death. Herein, we studied the protective effects of ginger on MDMA- induced apoptosis.

Methods: 15 Sprague dawley male rats were administrated with 0, 10 mg/kg MDMA, or MDMA along with 100mg/kg ginger, IP for 7 days. Brains were removed to study the caspase 3, 8, and 9 expressions in the hippocampus by RT-PCR. Data was analyzed by SPSS 16 software using the one-way ANOVA test.

Results: MDMA treatment resulted in a significant increase in caspase 3, 8, and 9 as compared to the sham group ($p<0.001$). Ginger administration however, appeared to significantly decrease the same ($p<0.001$).

Discussion: Our findings suggest that ginger consumption may lead to the improvement of MDMA-induced neurotoxicity.

1. Introduction

Ecstasy or 3, 4-methylenedioxymethamphetamine (MDMA), is a synthetic amphetamine derivative and an illicit drug of abuse which is primarily consumed by young people in dance and music environments. Many studies have demonstrated that MDMA is neurotoxic to the serotonin neurons (O'Leary et al., 2001). These effects seem to be dose- related, leading to memory impairment (Vorhees et al., 2009) and apoptosis in the hippocampus (Soleimani et al., 2012). It has been shown that MDMA induces cell death trough an apoptotic pathway through the release od cytochrome C and activation of the caspase cascade (Jiménez et al., 2004). The neurotoxicity associated with MDMA exposure may be the result of oxidative stress leading to the formation of hydroxyl radicals (Shankaran et al., 1999), lipid peroxidation (Alves et al., 2009) and an increase in the number of tunnel positive cells in the hippocampus. The imbalance between reactive oxygen species (ROS) and the internal antioxidants result in the oxidative stress. Glutathione is an important intracellular antioxidant that protects cells against oxidative stress (Franco and Cidlowski, 2012).

It has been repeatedly reported that MDMA treatment may decrease the glutathione concentration (Miranda et al., 2007).

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As glutathione is unable to cross the BBB, possible treatment options would include the glutathione analog as a suitable candidate for therapeutic applications (Slivka et al., 1987).

There is evidence that dietary components could improve brain damage and cognitive function (Bisson et al., 2008 and Head, 2009). Ginger, member of the family of zingiberaceae, is widely used as a spicy seasoning. Moreover, it is used in Asian traditional medicine for treatment of stomach ache (Mascolo et al., 1989), nausea, diarrhea, and joint and muscle pain (Ojewole et al., 2006).

Ginger's antioxidant activity (Nanjundaiah et al., 2009) and neuroprotective effects (Waggas et al., 2009) have been identified well. Kyung et al. suggest that ginger can reduce cell death and restore the motor function in rats with spinal cord injury (2006). Our aim was to investigate the effects of ginger on the caspase expression following MDMA treatment. Our hypothesis was if ginger has any anti apoptotic effect, then MDMA plus ginger treated rats should exhibit diminished caspase expression in their hippocampui.

2. Methods

2.1. MDMA and Ginger Preparation

3, 4-methylenedioxymethamphetamine was obtained from the Drug Control Headquarters. Zingiber officinale rhizomes with herbarium code no. 1483 were obtained from the Iranian institute of medicinal plant. About 500 g of dried rhizomes powder of Zingiber officinale were extracted with 3 liter 70% aqueous ethanol using percolation method at room temperature. The extracts were filtered through Whatman filter paper and evaporated to dryness under reduced pressure at a maximum of 40ºC using a rotary evaporator. Zingiber officinale yielded 33.28% dried extract. For treatment ginger was solved in normal saline.

2.2. Animals

We included 15 male Sprague Dawley rats, weighing 200-250 g (Razi Institute, Iran). Animals were allowed to acclimatize to the colony room for one week prior to administration of MDMA. Rats were kept in the colony room at a temperature of 21 ± 1°C, relative humidity of 55± 5%, on a 12-h light/12-h dark cycle with access to water and food ad libitum. All experimental procedures were performed in accordance with the Guidelines of the Ethical Committee of Tehran University of Medical Sciences.

Animals were assigned to the following groups:

1. Sham (saline) group (n=5) received normal saline ,1 ml/kg, intraperitoneally (i.p.), daily for 1 week

2. MDMA groups (n=5) received 10 mg/kg MDMA,i.p., daily for 1 week.

3. Treatment group (n=7) received 100 mg/kg ginger, i.p. at 9:00 plus 10 mg/kg MDMA at 13:00, i.p., daily for 1 week (Mehdizadeh et al., 2012).

2.3. RT-PCR Experiment

The day after the last treatment, animals were euthanized by cervical dislocation. The brains were removed; hippocampi were immediately dissected out on ice, and then frozen in liquid nitrogen and kept at -80 °C until use.

Total mRNA was extracted from the frozen hippocampi by using phenol-chloroform method. Tissue samples were homogenized in 1000 µl RNATM (Cinnagen, Tehran, Iran) then 200 µl ice-cold chloroform was added. The homogenates were centrifuged (Eppendrof, Hamburg, Germany) at 12000 g for 20 min in 4ºC. The RNA of the supernatant was precipitated with isopropanol, and washed with ethanol 75%. The air-dried RNA pellet was dissolved in RNase free water. cDNA first-strand synthesis was performed using a cDNA synthesis Kit (Quiagen, Hilden, Germany). First strand cDNA was used as template for subsequent PCR with a PCR master kit (Cinnagen, Tehran, Iran), and primers (Cinnagen, Tehran-Iran) as follow:

β-actin: forward 5TGGAGAAGAGCTATGAGCTGCCTG3

reverse 5GTGCCACCAGACAGCACTGTGTTG3

caspase3: forward 5TTTGGAACGAACGGACCTGT3

reverse 5CACGGGATCTGTTTCTTTGC3

caspase 8: forward GCAGGACATGTGGGACTCGCC 3

reverse 5 TCAGGCACAGGCACCGCTTTC 3

caspase 9 forward 5GAAGAACGACCTGACTGCTAAG 3

reverse 5 AGGAGACAAAACCTGGGAAG 3

The PCR reactions included initial denaturation at 95 ºC for 3 min, followed by 35 cycles with 95 ºC for 50 s, 58 ºC for 45 s and 72 ºC for 50 s for caspase3, and 9 and 35 cycles with 95 ºC for 50 s, 59 ºC for 45 and 72 ºC for

50 s for caspase 8. The reactions were terminated by 72 ºC for 7 min of elongation period. The same annealing temperature was used for β-actin. PCR products were separated by electrophoresis in 1% agarose gel at 100V. Our semi-quantitative analysis were made using a digital imaging system (UVIdoc,Houston,Texas,USA).

2.4. Statistical Analysis

Statistical analyses were performed using the SPSS 16 software. Data were presented as the mean ± S.E.M and the results were analyzed by one -way ANOVA with Tukey post-hoc comparison test. The $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Protective Effect of ginger on Caspase-3 Expression

As shown in Figure 1, there was a significant increase in caspase 3 expression in the MDMA group as compared to the sham group (P<0.001). Ginger pretreatment significantly decreased caspase 3 expression compared to the MDMA group (p<0.001).

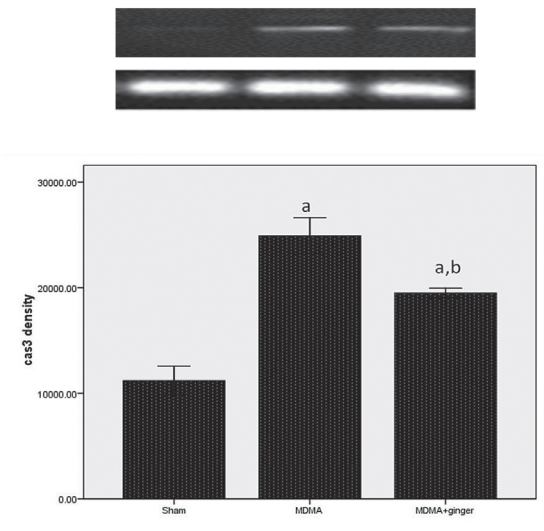

Figure 1. RT-PCR analysis of the caspase-3 expression in sham and treatment groups. A significant difference in the caspase-3 expression between sham and other groups was noted (ap<0.001 vs. sham). Administration of ginger down-regulated the caspase-3 expression (b p<0.001 vs. MDMA group).

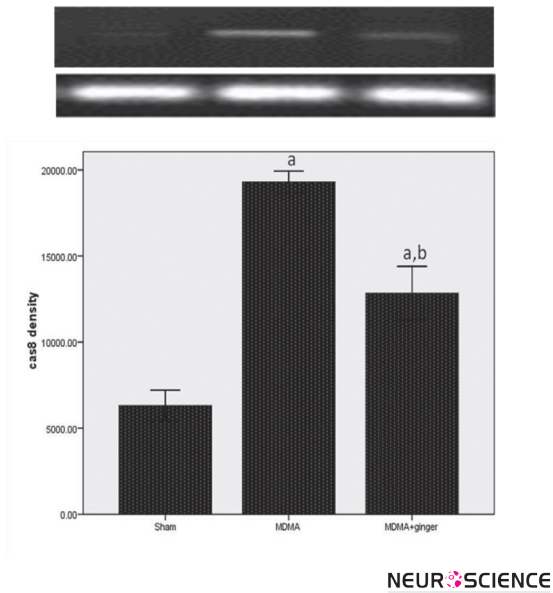

Figure 2. RT-PCR analysis of the caspase-8 expression in sham and treatment groups. MDMA treatment led to a significant increase in the caspae-9 expression (ap<0.001 vs. sham). Administration of ginger down-regulated the caspase-9 expression (b p<0.001 vs. MDMA group).

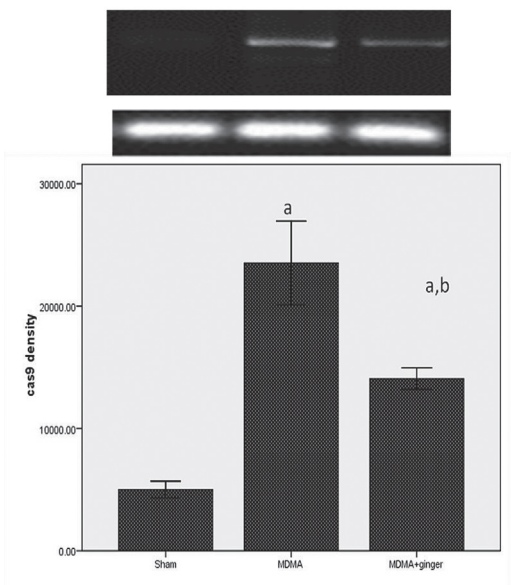

Figure 3. RT-PCR analysis of the caspase-9 expression in sham and treatment groups. Exposure to MDMA caused an increase in the caspae-8 expression (ap<0.001 vs. sham). Administration of ginger down- regulated the caspase-8 expression (b p<0.001 vs. MDMA group).

3.2. Protective Effect of Ginger On Caspase-8 Expression

With regards to the caspase 3 mRNA expression, there was significant difference between the MDMA and control group for caspase8 expression ($p < 0.001$, Figure 2). Moreover, subsequent analysis revealed that exposure to ginger resulted in significant reduction in caspase 8 expression compared to MDMA- treated rats ($p<0.001$).

3.3. Protective Effect of Ginger on Caspase-9 Expression

Densitometry from the electrophoresis gel showed significantly more expression of the caspase-9 gene in the MDMA group than the other groups ($P<0.001$, Figure 3). Ginger pretreatment caused a significant reduction in caspase-9 expression as compared to the MDMA group ($p<0.001$).

4. Discussion

The major finding of this study was the attenuation of caspase expression by ginger following MDMA treatment. Pretreatment with ginger was protective against MDMA- induced neurotoxicity in the hippocampus.

The brain is sensitive to oxidative stress due to low antioxidant and cell membrane lipids (Café et al., 1995). Therefore, the use of an external antioxidant is one of the most common therapeutic strategies for the treatment of neurotoxicity. Ginger is a spice that contains thiol group, which has antioxidant activity (Nanjundaiah et al., 2009) and neuroprotective effect (Waggas et al., 2009).

The hippocampus is an important brain structure substantially involved in learning and memory (Deng et al., 2012). Several studies have suggested that many neurotoxic factors can induce neuronal damage in the hippocampus (Che et al., 2010 and Singh et al., 2010), thus the use of external antioxidant may improve these insults (Alipanahzadeh et al., 2012, Soleimani et al., 2011).

Consistent to other study, our results demonstrated a well noted MDMA-induced neuronal death in the hippocampus (Wang et al., 2009 and Escubedo et al., 2011). MDMA causes rapid intracellular Ca2+ influx, mitochondrial membrane depolarization, ROS production and Caspase-9 activation (Montgomery et al., 2010). Injection of methamphetamine as another amphetamine derivates triggers the activation of the programmed cell death pathway and causes up-regulation of Bax and down regulation of Bcl-2 (Jayanthi et al., 2001).

Programmed cell death or apoptosis depends on activation of caspases such as caspases 3, 8, and 9 that cleave a number of substrates resulting in the biochemical and morphological changes typical for this form of death (For review see Favaloro et al., 2012).

In this study, we showed down- regulation of caspase 3, 8, and 9 genes in ginger plus MDMA treated group in comparison with MDMA group. Our results are consistent with the other studies. For instance, Mehdizadeh et al. reported that ginger could alter MDMA- induced apoptosis (Mehdizadeh et al., 2012). They showed that ginger pretreatment up- regulated anti apoptotic Bcl-2 and down- regulated proapoptotic Bax proteins in MDMA- treated rats. Furthermore, 6-shagoal purified from ginger could lead to prominent decrease in PARP apoptosis protein and increase in the expression of Bcl-2 antiapoptotic protein in spinal cord injury (Kyung et al., 2006). It has been reported that ginger can decrease the oxidative stress by increasing the activity of SOD in cerebral cortex, hippocampus, and striatum and increases the activity of CAT and GSH in cerebral cortex and hippocampus resulting in the decrease of lipid peroxidation level in all areas mentioned earlier (Kyung et al., 2006). Therefore the neuroprotective effect of Z.afficinale extract might be related to its antioxidant effects.

In conclusion, our findings suggested that ginger could decrease MDMA-induced apoptosis in the hippocampus of male rats. Therefore, ginger appears to be a useful medicinal herb as a potential treatment for the MDMA-associated adverse effects.

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References

Alipanahzadeh, H., Soleimani, M. , Soleimani Asl , S., Mehdizadeh, M. (2012). The Effect of TGF-alpha on Neurogenesis in Subventricular Zone of Rat Brain after Ischemia-Reperfusion. Journal of Basic and Clinical Neurosciences, 3(2),12-15.

Alves, E., Summavielle, T., Alves, C.J,, Custódio, J.B., Fernandes, E., De Lourdes Bastos, M., et al..(2012). Ecstasy-induced oxidative stress to adolescent rat brain mitochondria in vivo: influence of monoamine oxidase type A. Addict Biol, 14,185-193.

Bisson, J.F., Nejdi, A., Rozan, P., Hidalgo, S., Lalonde, R., Messaoudi, M.(2008). Effects of long-term administration of a cocoa polyphenolic extract (Acticoa powder) on cognitive performances in aged rats.Br J Nutr, 100,94-101.

Café, C., Torri, C., Bertorelli, L., Tartara, F., Tancioni, F., Gaetani, P.(1995).Oxidative events in neuronal and glial cell-enriched fractions of rat cerebral cortex.Free Radic Biol Med ,19, 853-857.

Che, Y., Wang, J.F., Shao, L., Young, T.(2010). Oxidative damage to RNA but not DNA in the hippocampus of patients with major mental illness.J Psychiatry Neurosci, 35,296-302.

Deng, W., Aimone, J.B., Gage, F.H. (2010). New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory?Nat Rev Neurosci, 11,339-350.

Escubedo, E., Abad, S., Torres, I., Camarasa, J., Pubill, D. (2011). Comparative neurochemical profile of 3,4-methylenedioxymethamphetamine and its metabolite alpha-methyldopamine on key targets of MDMA neurotoxicity.Neurochem Int, 58,92-101.

Favaloro B, Allocati N, Graziano V, Di Ilio C, De Laurenzi V. (2012). Role of apoptosis in disease. Aging (Albany NY), 4(5),330-349.

Franco, R., Cidlowski, J.A. (2012). Glutathione efflux and cell death.Antioxid. Redox. Signal. 15, 1694-1713.

Head, E. (2009). Oxidative damage and cognitive dysfunction: antioxidant treatments to promote healthy brain aging. Neurochem Res. 34,670-678.

Jayanthi, S., Deng, X., Bordelon, M., McCoy, M.T., Cadet, J.L. (2001). Methamphetamine causes differential regulation of pro-death and anti-death Bcl-2 genes in the mouse neocortex. FASEB J, 15,1745-1752.

Jiménez ,A., Jordà, E.G., Verdaguer, E., Pubill, D., Sureda ,F.X., Canudas, A.M., et al..(2004). Neurotoxicity of amphetamine derivatives is mediated by caspase pathway activation in rat cerebellar granule cells. Toxicol Appl Pharmacol, 15 ,223-234.

Kyung, K.S., Gon, J.H., Geun, K.Y., Sup, J.J., Suk, W.J., Ho, K.J.(2006). 6-Shogaol, a natural product, reduces cell death and restores motor function in rat spinal cord injury.Eur J Neurosci , 24,1042-1052.

Mascolo, N., Jain, R., Jain, S.C., Capasso, F. (1989).Ethnopharmacologic investigation of ginger (Zingiber officinale).J Ethnopharmacol, 27,129-140.

Mehdizadeh, M., Nejhadi, A., Fallah huseini, H., Choopani ,S., Mohammadzadeh Kazorgah, F.,Shekarriz, N. et al.. (2012). Zingiber fficinale alters 3, 4-methylenedioxymetham-induced neurotoxicity in rat's brain. Cell Journal Cell Journal(Yakhteh), 14 (3),177-184.

Miranda, M., Bosch-Morell, F., Johnsen-Soriano, S., Barcia, J., Almansa, I., Asensio, S. et al.. (2007).Oxidative stress in rat retina and hippocampus after chronic MDMA ('ecstasy') administration.Neurochem Res, 32(7),1156-1162.

Montgomery, T., Sitte, H., McBean, G.J. (2010). 4-Methylthioamphetamine (4-MTA) induces mitochondrial-dependent apoptosis in SH-SY5Y cells independently of dopamine and noradrenaline transporters. BMC Pharmacology, 10(Suppl 1),A22.

Nanjundaiah, S.M., Annaiah, H.N., Dharmesh, S. (2009) Gastroprotective Effect of Ginger Rhizome (Zingiber officinale) Extract: Role of Gallic Acid and Cinnamic Acid in H+, K+-ATPase/H. pylori Inhibition and Anti-oxidative Mechanism. Jul 1.

Ojewole, J.A. (2006).Analgesic, antiinflammatory and hypoglycaemic effects of ethanol extract of Zingiber officinale (Roscoe) rhizomes (Zingiberaceae) in mice and rats.Phytother Res, 20,764-772.

O'Leary, G., Nargiso, J., Weiss, R.D. (2001). 3, 4-methylenedioxymethamphetamine (MDMA): a review. Curr Psychiatry Rep, 3(6), 477-483.

Shankaran, M., Yamamoto, B.K., Gudelsky, G.A. (1999). Involvement of the serotonin transporter in the formation of hydroxyl radicals induced by 3,4-methylenedioxymethamphetamine.Eur J Pharmacol, 3,103-110.

Singh, M., Nam, D.T., Arseneault, M., Ramassamy, C. (2010) Role of by-products of lipid oxidation in Alzheimer's disease brain: a focus on acrolein.J Alzheimers Dis, 21,741-756.

Slivka, A., Mytilineou, C., Cohen, G.(1987). Histochemical evaluation of glutathione in brain.Brain Research. 21,275-284.

Soleimani Asl, S. , Falahati, P., Shekarriz, N., Molavi, N., Esmaeili, F. et al. (2011). Protective effects of N-acetyl-L-cystein on 3,4-methylene dioxymethamphetamie-induced neurotoxicity in cerebellum of male rats. Journal of Basic and Clinical Neurosciences, 3(1),45-48.

Soleimani Asl, S., Farhadi, M.H., Moosavizadeh, K., Samadi Kuchak Saraei, A., Soleimani, M. (2012). Evaluation of Bcl-2 family gene expression in hippocampus of 3-4 methylenedioxymethamphetamine treated rats.Cell Journal, 13,275-280.

Vorhees, C.V., Schaefer, T.L., Skelton, M.R., Grace, C.E., Herring, N.R., Williams, M.T.(2009). (+/-)3,4-Methylenedioxymethamphetamine (MDMA) dose-dependently impairs spatial learning in the morris water maze after exposure of rats to different five-day intervals from birth to postnatal day twenty. Dev Neurosciences, 31:107-120.

Waggas, A.M.(2009). Neuroprotective evaluation of extract of ginger (Zingiber officinale) root in monosodium glutamate-induced toxicity in different brain areas male albino rats.Pak J Biol Sci, 12:201-212.

Wang, X., Zhu, S.P., Kuang, W.H., Li, J., Sun, X., Huang, M.S,, Sun, X.L. (2009). Neuron apoptosis induced by 3,4-methylenedioxy methamphetamine and expression of apoptosis-related factors in rat brain. Sichuan Da Xue Xue Bao Yi Xue Ban, 40,1000-1002, 1037.

Intracerebroventricular Injection of Lipopolysaccharide Increases Gene Expression of Connexin32 Gap Junction in Rat Hippocampus

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ABSTRACT

Introduction: Gap junctions are intercellular membrane channels that provide direct cytoplasmic continuity between adjacent cells. This communication can be affected by changes in expression of gap junctional subunits called Connexins (Cx). Changes in the expression and function of connexins are associated with number of brain neurodegenerative diseases. Neuroinflammation is a hallmark of various central nervous system (CNS) diseases, like multiple sclerosis, Alzheimer's disease and epilepsy. Neuroinflammation causes change in Connexins expression. Hippocampus, one of the main brain regions with a wide network of Gap junctions between different neural cell types, has particular vulnerability to damage and consequent inflammation. Cx32 – among Connexins– is expressed in hippocampal Olygodandrocytes and some neural subpopulations. Although multiple lines of evidence indicate that there is an association between neuroinflammation and the expression of connexin, the direct effect of neuroinflammation on the expression of connexins has not been well studied. In the present study, the effect of neuroinflammation induced by the Lipopolysaccharide (LPS) on Cx32 gene and protein expressions in rat hippocampus is evaluated.

Methods: LPS (2.5µg/rat) was infused into the rat cerebral ventricles for 14 days. Cx32 mRNA and protein levels were measured by Real Time PCR and Western Blot after 1st, 7th and 14th injection of LPS in the hippocampus.

Results: Significant increase in Cx32 mRNA expression was observed after 7th injection of LPS ($P<0.001$). However, no significant change was observed in Cx32 protein level.

Conclusion: LPS seems to modify Cx32 GJ communication in the hippocampus at transcription level but not at translation or post-translation level. In order to have a full view concerning modification of Cx32 GJ communication, effect of LPS on Cx32 channel gating should also be determined.

1. Introduction

Gap junctions are specialized cell-cell contacts between eukaryotic cells, composed of aggregates of transmembrane channels, which directly connect the cytoplasm of adjacent cells, allowing intercellular movement of small molecular weight molecules (up to 1 KDa) including ions, metabolites and second messengers (Condorelli et al., 2003; Sohl et al., 2005). Each channel consists of two hemi-

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channels (termed connexons), each of which is composed of six subunit proteins called connexin (Cx) (Sohl et al., 2005). A total of 21 Cx family members have been identified in the mammalian genome (Kielian, 2008). Gap junctions facilitate ionic homeostasis and synchronization of action potential in the nervous system (Sohl et al., 2000). Alteration in the expression and the function of connexins are associated with several of brain pathologies and neurodegenerative diseases, suggesting that they could contribute to the expansion of brain damages (Rouach et al., 2002). Some studies have provided evidence that gap-junctional communication is associated with the spread of cell death signals, while others have equally demonstrated neuroprotective effects (Cronin et al., 2008; Froger et al., 2009, 2010). Enhanced gap junctional coupling is proposed as a possible mechanism underlying neuronal synchronization (Li et al., 2001). Gap junction coupling can be regulated at several levels including alteration in Cx transcription, translation, stability, post translational processing and channel gating (Saez et al., 2003; Garg et al., 2005).

Inflammation is a hallmark of various CNS diseases such as bacterial and viral infections and cerebral ischemia (Garg et al., 2005). Brain injuries as well as neurodegenerative diseases, are associated with neuroinflammation (Froger et al., 2009). Alterations in Cx expression have been associated with neuroinflammation (Garg et al., 2005). However, the direct effects of neuroinflammation on the gene and protein regulation and expression of connexins as building blocks of gap junctions has not been fully characterized.

One of the main brain regions with a wide network of Gap junctions between different neural cell types is hippocampus, which has particular vulnerability to damage due to hypoglycemia, ischemia/hypoxia, trauma and subsequent Neuroinflammation (Sohl et al., 2000; Zeinieh et al., 2010; Karpuk et al., 2011). Among Cxs, Cx32 – which is generally expressed in Olygodandrocytes and some neural subpopulations – is well represented throughout the CNS (Sohl et al., 2000; Bennett et al., 2004). Cx32 in hippocampus is predominantly expressed in oligodendrocytes (oligodendrocyte/ oligodendrocyte or oligodendrocyte/astrocyte GJs) and parvalbumin-positive inhibitory interneurons of CA1 subfield (Rouach et al., 2001; Rash et al., 2002). There is no report regarding changes of this Cx during neuroinflammation.

The bacterial Endotoxin Lipopolysaccharide (LPS) is a stimulator of microglia and is used extensively as a model of neuroinflammation (Turrin et al., 2001; Kovacs et al., 2006).

The present study was undertaken to examine the changes of Cx32 mRNA and protein expression in rat hippocampus consequent to acute and chronic intracerebroventricular (i.c.v) injection of LPS.

2. Methods

2.1. Animals

Male Wistar rats (280–320 g, Institute Pasteur of Iran) were used in this study. The animals were housed in standard Plexiglas cages with free access to food (standard laboratory rodent's chow) and water. The animal house temperature was maintained at 23 ± 1.0 °C with a 12-h light/dark cycle (light on from 6.00 a.m.). All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) in such a way to minimize the number of animals used and their suffering. Each animal was tested once.

2.2. Materials

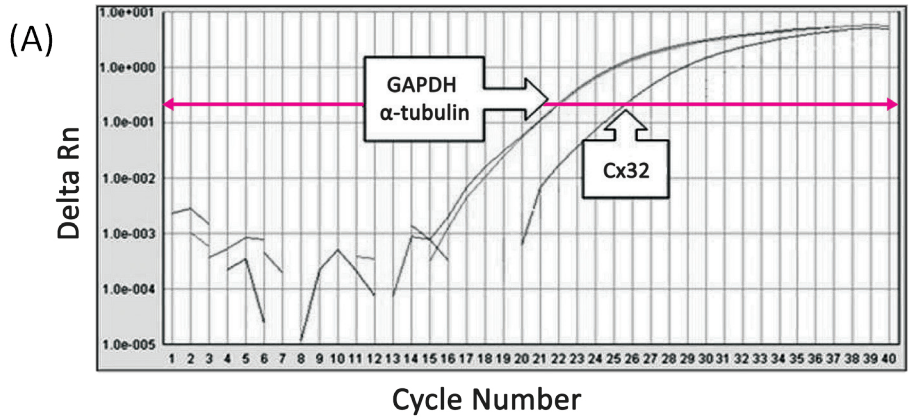
Ketamine (Rotex Medica, Germany), Xylazine (Chanelle, Ireland), LPS (Escherichia coli serotype 026:B6, Sigma, UK), RNX-Plus Reagent, Agarose, Acrylamide (CinnaGene, Iran) Quantitect Reverse Transcriptase kit (Qiagen, Germany), Power Syber Green PCR Master Mix, Rox and dNTP mix (Warrnington, UK) Page Ruler™ Prestained Protein Ladder and Protein Loading Buffer Pack (Fermentas, Lithuania), Protease Inhibitor Cocktail (Roche, Germany), Enhanced Chemiluminescence (ECL) Advance Western Blotting Detection Kit (Amersham, UK). Monoclonal anti-connexin 32, Monoclonal anti-α-tubulin and anti-mouse IgG peroxidase conjugates (Sigma-Aldrich, USA) were used in this study. LPS was dissolved in phosphate buffer solution (PBS) and was prepared freshly on the day of use.

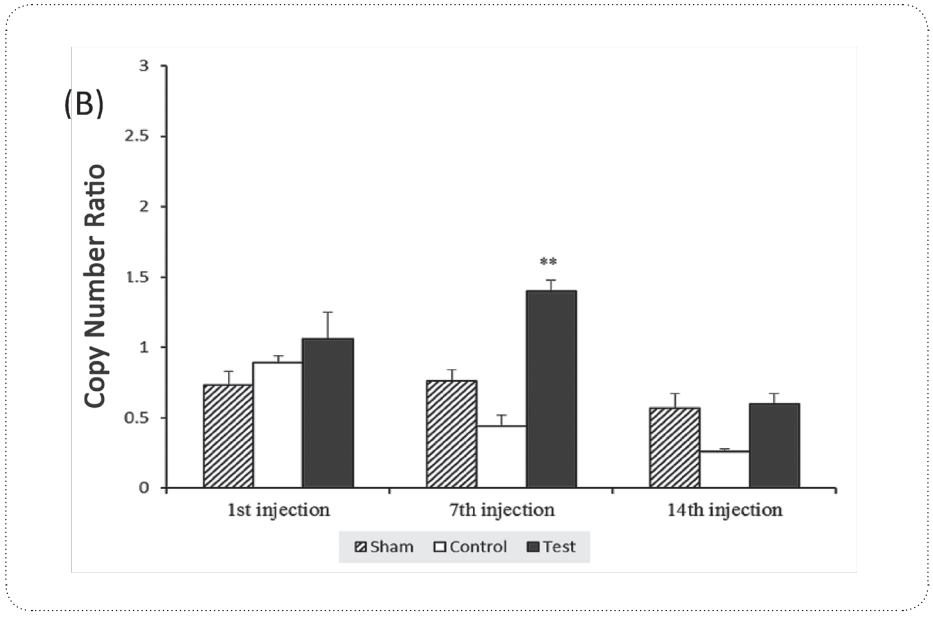
2.3. Stereotaxic Surgery and LPS Injection

The rats were stereotaxically implanted with a cannula in the left lateral ventricle (Paxinos & Watson, 2007). The animals were given 7 days recovery after surgery, before the injection protocol was started. LPS at the dose of 2.5 µg/rat was infused once daily i.c.v. for 14 days. For each experimental group, a sham (cannula-implanted non-injected) and a control (cannula-implanted phosphate buffer (PBS)-injected) groups were considered.

2.4. Tissue Collection

To measure changes in Cxs mRNA expression, the hippocampi were dissected 24 h after 1st, 7th and 14th in-





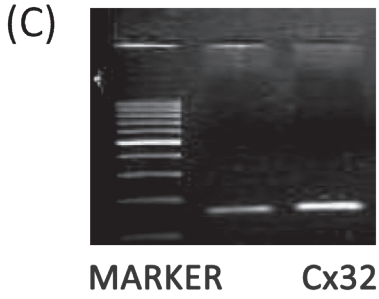


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Figure 1 (A). Amplification plots of the target and reference genes (Cx32, α-Tubulin, GAPDH) in the Real-time PCR assay. The amplification curves of the both reference genes have crossed the threshold line at the same point. (mCt) Mean threshold cycle. (mCt GAPDH and α-Tubulin=21.95, mCt Cx32=25.61). (B) Cx32 mRNA level in the hippocampus of the rats after daily intracerebroventricular injection of LPS. Connexin mRNA level was normalized to α-tubulin and GAPDH mRNA level. Data are expressed as means ± S.E.M (n=5). ** $p<0.001$ compared to respective control group. (C) Denaturing agarose gel electrophoresis to evaluate samples for other DNA contamination during RT-PCR reaction and also proves the integrity of the samples in RNA extraction process.

jections of LPS. All the animals and their corresponding controls were decapitated under deep Ether anesthesia and their brain were removed immediately. The brains were incubated in chilled artificial cerebrospinal fluid (ACSF) with pH 7.3 consisted of the following composition (in mM): 124 NaCl, 4.4 KCl, 2 CaCl2, 2 MgCl2, 1.2 KH2PO4, 25 NaHCO3 and 10 Glucose. The hippocampus of the brains were removed and frozen immediately in liquid nitrogen and stored at -80°C. The rest of the brains were placed in 10% formalin for at least 3 days at room temperature and they were then processed, cut into 10µm thick slices and qualitatively examined for cannula position using a stereoscopic microscope (Olympus, Japan). The data of the animals, in which the cannula was in the false place, were not included in the results.

2.5. Gene Expression Assay

Tissue Preparation: The frozen hippocampus samples were pulverized completely and mixed with 200 µl of chilled phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4.7H2O, and 1.4 mM KH2PO4), vortexed for 30 sec then spinned and aliquoted in two micro tubes equally. One of so prepared samples was used for gene expression study and the second part for immunoblotting. An appropriate volume of a protease inhibitor cocktail according to manufacturer's proposal was added to samples, which were allocated for immunoblotting.

RNA Extraction: Total cellular RNA was isolated from the hippocampus by a modification of the guanidine Isothiocyanate Phenol-Chloroform method (Ausubel et al., 2002) using RNX-PLUS reagent. The RNA was treated with 10U RNase free DNase I (Roche, Germany) to avoid any DNA contamination (Fig 1C). The concentrations and purity of the RNAs were determined by spectrophotometry (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA). The mean absorbance ratio at 260/280 nm and 260/230 nm were 1.94 ± 0.0 and 1.98 ± 0.1, respectively.

cDNA Synthesis: The reverse transcription reaction was performed with first strand cDNA synthesis kit (Roche, Germany) using Oligo-dT primer, AMV reverse transcriptase and 1µg total RNA as template, according to the manufacturer's instructions. The concentration of synthetic cDNA was measured using NanoDrop ND-1000 Spectrophotometer at 260 and 280 nm. DNA samples with the A260/A280 ratios higher than 1.5 were selected for quantitative analysis.

Real Time PCR and Comparative Threshold Cycle Method: Cx32 was chosen as target gene and α-tubulin and GAPDH were used as internal reference genes. All primers (Table 1) were designed using primer express software v.3.0 (Applied Biosystems, Foster City, CA, USA). The specificity of the primers for their target sequences was checked on NCBI website (www.ncbi.nlm.nih.gov/blast). SYBR Green I real time PCR assay was carried out in final reaction volumes of 25 µL with 12.5 µL of SYBR Green I Master mix (Applied Biosystems, Warrington, UK), 100nM of forward and reverse primers and 300ng of cDNA. Thermal cycling was performed on the ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the following cycling conditions: 10 min at 95ºC as first denaturation step, followed by 40 cycles at 95ºC for 15 sec and 60ºC for 1 min. Each complete amplification stage was followed by a dissociation stage at 95 ºC for 15 sec, 60 ºC for 30 sec and 5 ºC for 15 sec. The extent of gene expression was calculated using comparative threshold cycle. The mean threshold cycle (mCT) was obtained from duplicate amplifications during the exponential phase of amplification. Then, mCT of reference genes were subtracted from mCT value of the target genes to obtain ΔCT. ΔΔCT values of each sample was calculated from corresponding CT values where ΔΔCT = [mCT target (control sample) - mCT reference (control sample)] - [mCT target (test sample) - mCT reference (test sample)]. The calculated ΔΔCT was converted to ratio using the ratio formula (Ratio = 2_ΔΔCT) (Livak & Schmittgen, 2001). Dissociation curve analysis was performed for each amplification reaction to detect any possible primer dimmers or non-specific PCR product (Ruiz-Ponte et al., 2006). Before using comparative threshold cycle method, amplification efficiency of each gene was determined from the standard curve drawn by plotting the logarithmic input amount of template DNA versus the corresponding CT values. The corresponding real time PCR efficiencies were calculated according to the slope of the standard curve and the following equation: Efficiency = [10(-1 / Slope)] - 1 (Vaerman et al., 2004). Data evaluation was carried out using the ABI PRISM 7300 Sequence Detection System and the SDS software v.1.2.3 (Applied Biosystems, UK).

2.6. Immunoblotting

The second part of the homogenized hippocampus tissues was removed from−80 °C and centrifuged at 12,000 g, 4 °C for 10 min. The supernatant was collected and total protein concentration was determined using Bio-Rad DC protein assay reagents. Samples were dissolved in protein loading buffer and denatured for 5 min at 95

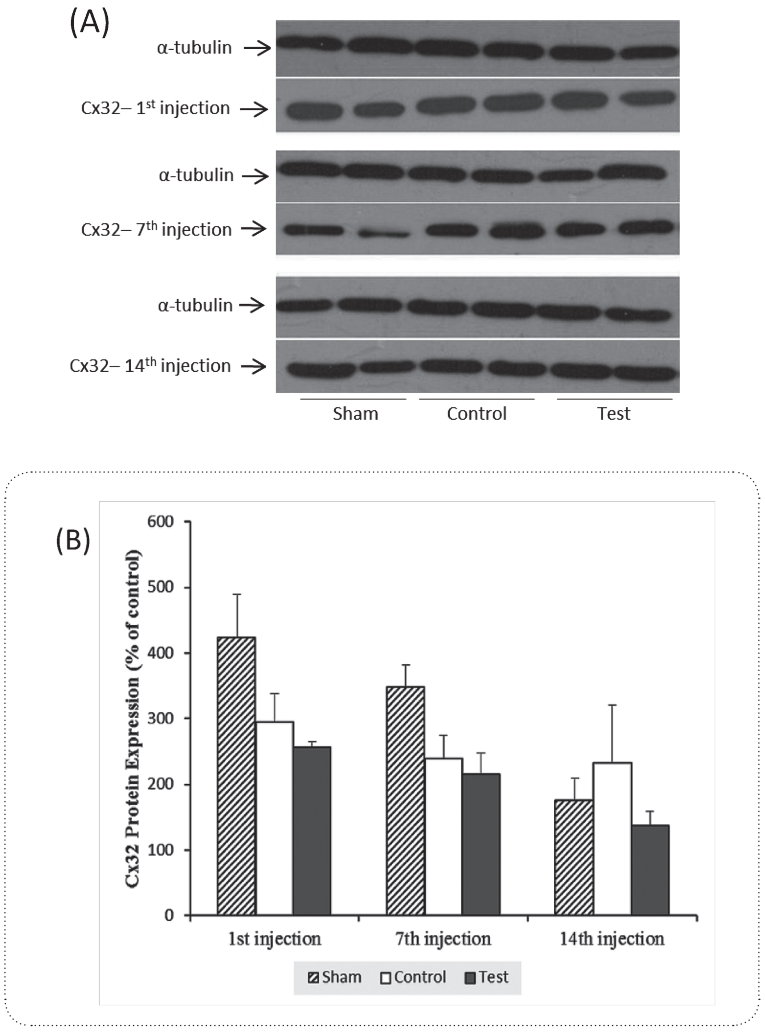

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Figure 2 (A). Immunoblots of Cx32 (32KDa) and α-tubulin (50KDa) for prepared samples. Each immunoblotting was performed in duplicate to increase the reliability of the measurements. (B) Cx32 protein level in the hippocampus of the rats after daily intracerebroventricular injection of LPS. Connexin protein level was normalized to α-tubulin protein level. Data are expressed as means ± S.E.M (n=5) compared to respective control group.

°C prior to loading. Equal amounts of protein from each animal (5 µg per lane for α-tubulin, 10 µg per lane for Cx32) were resolved by denaturing SDS-Polyacrylamide gel electrophoresis (SDS-PAGE), 12% acrylamide and transferred to a PVDF membrane (Roche, Germany) by electroblotting (Mini trans blot electrophoretic transfer cell, Bio-Rad). The membrane was blocked in TBST buffer (100 mMTris base, 150 mMNaCl, and 0.2% Tween 20) containing 2% ECL Advance blocking agent at room temperature for 60 min, rinsed briefly with TBST buffer and then incubated for 60 min with the following primary antibodies: mouse monoclonal anti-connexin 32 diluted 1:200,000, and mouse monoclonal anti-α-tubulin diluted 1:200,000. The antibodies were diluted in blocking buffer. After washing with TBST buffer 4 times (1× for 15 min and 3× for 5 min), the membrane was incubated with peroxidase conjugated goat anti-mouse IgG (diluted 1:100,000 and 1:2,000,000 for Cx32 and α-tubulin, respectively) for 1 h, then washed with TBST buffer 4 times (1× for 15 min and 3× for 5 min) and reacted with ECL Advance western blotting detection reagents, for 4 min. An X-ray film (Retina, USA) was used for 30s to 10min and then developed to visualize the antibody binding (Fig 2A). Bands were quantified by densitometry using Labworks analyzing software (Ultra Violet Products, U.K). The relative levels of Cx32 proteins were expressed as ratios (Cx32/α-tubulin×100).

2.7. Statistical Analysis

The data were analyzed by ANOVA with Tukey post hoc test and presented as mean ± S.E.M. In all experiments, $P<0.05$ was considered statistically significant.

Table 1. The characteristics of the primers used in the Real Time PCR assay

Gene	Sequence	GC%	Melting Temperature °c	Primer Length
Cx32-Forward	CGGCATCTGCATTATCCTCAAC	50%	60.4	22
Cx32-Reverse	CAGCAGCTTGTTGATCTCATTCTG	46%	60	24
α-Tubulin–Forward	CTGGAACCCACAGTTATTGATGAAG	45%	59.8	25
α-Tubulin-Reverse	GGCATAGTTATTGGCAGCATCCTTC	45.8%	60	24
GAPDH-Forward	AGTCAAGGCTGAGAATGGGAAG	50%	58.5	22
GAPDH-Reverse	CATACTCAGCACCAGCATCACC	54.6%	59.2	22

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3. Results

3.1. Elevation of Cx32 mRNA Levels in LPS-Treated Rats

Melting curve analysis for Cx32, GAPDH and α-tubulin gene fragments revealed unique PCR product in each reaction. Each peak represented a unique PCR product in each reaction. Melting temperature of 80.5 °C for GAPDH, 81.3 °C for α-tubulin and 79.0 °C for Cx32 were obtained. The amplification curves of the both reference genes (α-tubulin, GAPDH) have crossed the threshold line at the same point. Mean threshold cycle of 21.95 for GAPDH and α-tubulin and 25.61 for Cx32 were obtained. (Fig 1A)

mRNA level of Cx32 significantly increased after 7th injection of LPS relative to the control group (n=5, $P<0.001$). But no meaningful change was detected after 1st and 14th injection of LPS in Cx32 mRNA expression compared to control group (Fig 1B).

3.2. Effect of LPS on Cx32 Protein Expression

Data from immunoblotting of Cx proteins showed that, there were no significant changes in the levels of Cx32 protein during acute and chronic injection of LPS in rat Hippocampus. Although some decreases in Cx32 protein expression was observed after LPS injection that was not statistically significant compared to control (n=5, $P>0.05$) (Fig 2B).

4. Discussion

Our data indicate a significant increase in Cx32 mRNA expression after chronic injection of LPS. However, no significant changes were observed in Cx32 protein abundance during this period. To our knowledge, this is the first in vivo study on the effect of LPS on hippocampal expression of Cx32 GJs in experimental animals.

Alterations in Cx expression are well recognized following neuroinflammation conditions (Garg et al, 2005). The GJ protein Cx32 is abundantly expressed in mammalian brain. Cx32-deficient mice display enhanced intrinsic excitability and dysfunction of inhibitory synaptic transmission in the neocortex (Suton et al., 2000). In the hippocampus, Cx32 is expressed predominantly in oligodendrocytes (Rash et al., 2001) and parvalbumin-positive inhibitory interneurons of CA1 subfield (Oguro et al., 2001). The expression of Cx32 GJ protein increases selectively in the CA1 GABAergic interneurons after global ischemia. Moreover, transgenic Cx32-null mice exhibit enhanced vulnerability to global ischemia-induced neuronal death, consistent with the role of Cx32 gap junctions in neuroprotection against ischemia-induced cell death (Oguro et al., 2001). These observations together with our findings in the present study – up-regulating of Cx32 mRNA expression after chronic seven days by LPS central injection – suggest that Cx32 gap junctions is overexpressed under brain damages as a part of adaptive processes in order to reduce damages and protect the hippocampal neurons. However, we could not detect any changes in hippocampal Cx32 protein expression during LPS injection period. It can be suggested that LPS has no effect on regulation of Cx32 expression at translational level. Our observation might be related to rapid turnover of Cxs protein (Kielian. 2008). Moreover, LPS might affect the stability of Cx proteins as reported by some researchers (Chanson et al., 2005). Indeed, the half-life of Cx32, the main Cx in the liver, is reduced during liver inflammation induced by LPS (De Maio et al., 2000). The lack of correlation between mRNA and protein level of Cxs observed in our study, is reported by other researchers as well. For instance, Oguro et al. (2001) found that global ischemia induces a marked reduction in Cx32 mRNA abundance and at the same time a marked increase in Cx32 protein level in mouse hippocampus.

Intercellular communication through GJs can be regulated at several levels including changes in Cx transcription, translation, stability, post translational processing, insertion/removal from the cell membrane and channel gating (Kielian. 2008). However, post-translational

processes are thought to be the major factor in regulating Cxs levels and functional coupling. Therefore, our results suggest no functional modulation of Cx32 GJ coupling by LPS. Nevertheless, effect of LPS on Cx32 channel gating should be determined to further elucidate effect of LPS on GJC.

In conclusion, the chronic central injection of LPS, upregulates Cx32 GJs in rat hippocampus at mRNA; however it does not affect the protein level. Evaluation of the expression changes of these Cxs in other models of neuroinflammation is required to clarify the role of Cx32 GJs in pathology of CNS inflammation and disease.

References

Ausubel, F. M., Brent, R., Kingston, R. E., et al. (2002). Short Protocols in Molecular Biology. (5th ed.). Wiley, New York.

Bennett, M.V.L. and Zukin, R.S. (2004). Electrical coupling and neuronal synchronization in the mammalian brain. Journal of Neuron. (41) 495-511.

Chanson, M., Derouette, J, P., Roth, I., Foglia, B., Scerri, I., Dudez, T., Kwak, B, R. (2005). Gap junctional communication in tissue inflammation and repair. Biochim Biophys Acta, (1711)197-207.

Condorelli, D, F., Trovato-salinaro, A., Mudo, G., Mirone, M. B., Belluardo, N. (2003). Cellular expression of connexins in the rat brain: neuronal localization, effects of kainite-induced seizures and expression in apoptotic neuronal cells. Euro Journal of Neurosci. (18) 1807-27.

Cronin, M., Anderson, P. N., Cook, J. E., Green, C. R., Becker, D. L. (2008). Blocking connexin43 expression reduces inflammation and improves functional recovery after spinal cord injury. Journal of Mole and Cell Neurosci. (39) 152-160.

De Maio, A., Gingalewski, C., Theodorakis, N, G,. Clemens, M, G. (2000). Interruption of hepatic gap junctional communication during acute inflammation. Shock, (14):53-59.Froger, N., Orellana, J. A., Calvo, C. F., Amigou, E., Kozoriz, M. G., Naus, C. C., et al. (2010). Inhibition of cytokine-induced connexin43 hemichannel activity in astrocytes is neuroprotective. Journal of Mole and Cell Neurosci. (45) 37-46.

Froger, N., Orellana, J. A., Cohen-Salmon, M., Ezan, P., Amigou, E., Saez, J. C. (2009). Cannabinoids prevent the opposite regulation of astroglial connexin43 hemichannels and gap junction channels induced by pro-inflammatory treatments. Journal of Neurochem. (111) 1383-1397.

Garg, S., Syed, M. M., Kielian, T. (2005). Staphylococcus aureus-derived peptidoglycan induces Cx43 expression and functional gap junction intercellular communication in microglia. Journal of Neurochem. (95) 475-483.

Karpuk, N., Burkovetskaya, M., Fritz, T., Angle, A., Kielian, T. (2011). Neuroinflammation Leads to Region-Dependent Alterations in Astrocyte Gap Junction Communication and Hemichannel Activity. Journal of Neuroscience. (31) 414-425.

Kielian, T. (2008). Glial connexins and gap junctions in CNS inflammation and disease. Journal of Neurochem. (106) 1000-1016.

Kovacs, Z., Kekesi, K. A., Szilagyl, N., et al. (2006). Facilitation of spike-wave discharge activity by lipopolysaccharides in Wistar Albino Glaxo/Rigswijk rats. Journal of Neurosci. (140) 731-742.

Livak, K. J., Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) Method. Methods. (25) 402–8.

Oguro, K., Jover, T., Tanaka, H., Lin, Y. Takashi, K., Oguro, N., et al. (2001). Global ischemia-induced increases in the gap junctional proteins connexin 32 (Cx32) and Cx36 in hippocampus and enhanced vulnerability of Cx32 knock-out mice. Journal of Neurosci. (21) 7534-7542

Paxinos, G., Watson C, R. The Rat Brain in Stereotaxic Coordinates. (2007). (6th ed.). Elsevier, San Diego.

Rash, J. E., Yasumura, T., Davidson, K. G., Furman, C. S.,Dudek, F. E, Nagy, J. I. (2001). Identification of cells expressing Cx43, Cx30, Cx26, Cx32, and Cx36 in gap junctions of rat brain and spinal cord. Journal of Cell Commun Adhes. (8) 315-320.

Rouach, N., Avignone, E., Meme, W., et al. (2002). Gap junctions and connexin expression in the normal and pathological central nervous system. Journal of Biol cell (94) 451-75.

Ruiz Ponte, C., Carracedo, A., Barros, F. (2006). Duplication and deletion analysis by Fluorescent real time PCR based genotyping. Journal of Clin Chem Acta. (363) 138-146.

Saez, J. C., Berthoud, V. M., Branes, M. C., Martinez, A. D., Beyer, E. C. (2003). Plasma membrane channels formed by connexins: their regulation and functions. Journal of Physiol. Rev. (83) 1359–1400.

Sohl, G., Guldenagel, M., Beck, H., et al. (2000). Expression of connexin genes in hippocampus of kainite-treated and kindled rats under conditions of experimental epilepsy. Journal of Mol Brain Res. (83) 44-51.

Sohl, G., Maxeiner, S., Willecke, K. (2005). Expression and functions of neuronal gap junctions. Journal of Nat Rev Neurosci. (6) 191-200.

Sutor, B., Schmolke, C., Teubner, B., Schirmer, B., Willecke, K. (2000). Myelination defects and neuronal hyperexcitability in the neocortex of connexin32-deficient mice. Journal of Cereb Cortex. (10) 684-697.

Turrin, N. P., Gayle, D., Ilyin, S. E., et al. (2001). Pro-inflammatory and anti-inflammatory cytokine mRNA induction in the periphery and brain following intraperitoneal administration of bacterial lipopolysaccharide. Brain Res. Bull. (54) 443-453.

Vaerman, J. L., Saussoy, P., Ingargiola, I. (2004). Evaluation of real-time PCR data. Journal of Biol Regul Homeost Agents. (18) 212–4.

Zeinieh, M., Talhouk, R., Marwan, E. (2010). Differential expression of hippocampal connexins after acute hypoxia in the developing brain. Journal of Brain and Development. (32) 810-817.

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The Effect of Food Deprivation on Nociception in Formalin Test and Plasma Levels of Noradrenaline and Corticosterone in Rats

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ABSTRACT

Introduction: The concentration of noradrenalin and corticosterone as the two nociception modulators change after fasting or stress situation. The aim of present study was to investigate the effect of food deprivation on formalin-induced nociceptive behaviours and plasma levels of noradrenalin and corticosterone in rats.

Methods: Food was withdrawn 12, 24 and 48 h prior to performing the formalin test, but water continued to be available ad libitum. The formalin solution (50 µL, 2%) was injected into plantar surface of hind paw. The nociception responses of the animals during the first phase (1-7 minutes), the inter-phase (8-14), the phase 2A (15-60) and the phase 2B (61-90) was separately evaluated. The plasma concentrations of noradrenalin and corticosterone were measured using specific ELISA and IRA kits, according to manufacturer's instructions.

Results: In contrast to the increasing of 48 h food deprived animals during phase 2, the nociceptive behaviours of 12 and 24 h groups decreased through the interphase, phase 2A and phase 2B. The injection of formalin in the normal male rats significantly decreased the plasma level of noradrenalin and corticosterone. Food deprivation for 12 and 24 h increased noradrenalin level significantly in comparison with control group which has caused by fasting induced antinociceptive behaviours. There was no significant change in food deprivation for 48 h group. Food deprivation for 12, 24 and 48 h had no effect on corticosterone level in male rats.

Discussion: The present study emphasizes that the acute food deprivation diminished the nociceptive behaviours in the formalin test and show a correlation with increase in plasma noradrenalin level.

1. Introduction

Both short-term and intermittent food deprivation are well known to have antinociceptive effect, which several neuromodulatory systems such as endogenous opioid system and adrenocortical hormones are known to be involved (Bodnar, Romero, & Kramer, 1988; Hamm & Knisely, 1986). Norepinephrine participates in descending pain inhibitory system. Brainstem nuclei A1–A7 such as locus coeruleus in centrally and sympathetic nerves in peripherally are the main sources of norepinephrine. Locus coeruleus in the pons has most projection to the dorsal horn of spinal cord (Proudfit, 1988; Kwiat & Basbaum, 1992) and has a key role in noradrenergic pain modulation. Locus coeruleus stimulation releases norepinephrine (Hentall, Mesigil, Pinzon, & Noga, 2003) and produces analgesia that is prevented by alpha-2-adrenoceptor antagonist's administration (Jones, 1991; Proudfit, 1988). Furthermore, norepinephrine is released by peripheral noxious stimulation (Takagi, Shiomi, Kuraishi, Fukui, & Ueda, 1979; Tyce &

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Yaksh, 1981; Yaksh & Tyce, 1981). Destruction of noradrenergic system decreased formalin-induced nociceptive behaviours in phase2 (Martin, Gupta, Loo, Rohde, & Basbaum, 1999a). Safari et al. indicated that chemical stimulation or inactivation of lateral hypothalamus induced analgesia effect and administration of lidocaine into the LC blocked the carbachol-induced analgesia (Safari, Haghparast, & Semnanian, 2009). Mustonen et al. in 2005 demonstrated that 3 and 7 days of wintertime food starvation in the male American mink decreased the plasma noradrenaline and corticosterone concentrations, respectively (Mustonen, Saarela, Pyykonen, & Nieminen, 2005a), but they observed brief increase, but not significant, in the noradrenalin concentrations after 48 h of food deprivation.(Mustonen, Saarela, Pyykonen, & Nieminen, 2005b). On the other hand, formalin injection elevated the noradrenaline concentration in the locus coeruleus which might be due to the pain induced by formalin (Sajedianfard, Khatami, Semnanian, Naghdi, & Jorjani, 2005b). In addition, noxious stimuli, such as foot shock as well as electrical stimulation of the locus coeruleus, accelerate noradrenaline turnover in the cerebral cortex (Singewald, Kaehler, & Philippu, 1999b). There are many evidence that showed norepinephrine is involved in pain modulation during formalin-induced nociceptive behaviors. For example, alpha-1-adrenoceptor binding was attenuated in the spinal dorsal horn during formalin-induced nociceptive behaviours in mice (Nalepa et al., 2005).

Administration of noradrenaline into the central grey before and at the end of short term food deprivation, couldn't change pain threshold (Bhunia, Bharambe, Singh, Premendran, & Pande, 2000). In men, long-term food deprivation increased release of adrenaline (Uvnas-Wallenstein & Palmblad, 1980). Based on the above literatures, indicating that fasting affects formalin-induced nociceptive behaviors and noradrenaline concentration, it can be hypothesized that food deprivation in a time-dependent manner modulates formalin induced nociceptive behaviors and it might be correlated to pain modulation of noradrenalin and corticosterone after food deprivation. To test this hypothesis, formalin tests were performed following 12, 24 and 48hr food deprivation and noradrenalin and corticosterone levels were measured in male rats.

2. Methods

2.1. Subjects

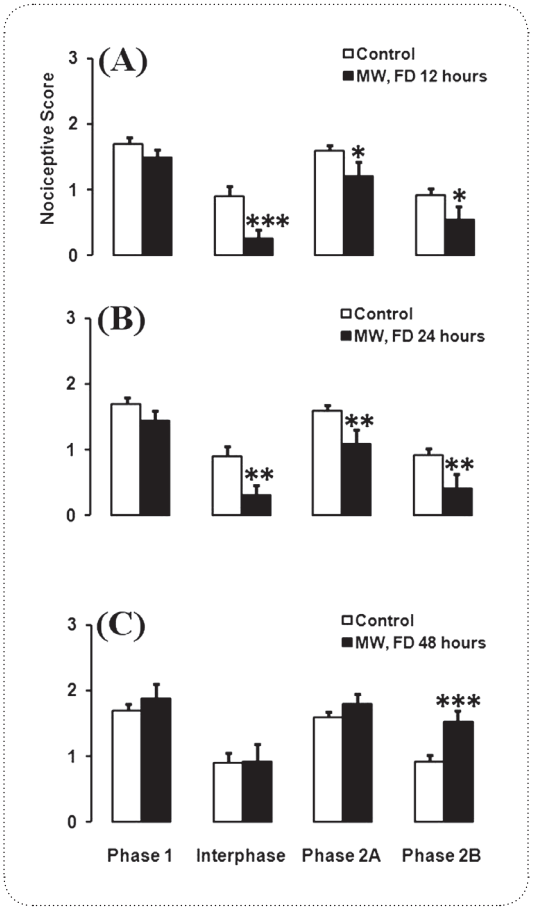
All experiments were done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996) and were approved by the Research and Ethics Committee of Qazvin University of Medical Sciences, Qazvin, Iran. Efforts were made throughout the experiments to minimize the animal discomfort and to reduce the number of animals used. Adult male and female Wistar rats (220–300 g) were purchased from Razi Institute (Hesarak Karj, Iran), and were housed in groups of three in a temperature controlled room, under a 12 h light–dark cycle with lights on at 7:00 to 19:00. Food and water were provided ad libitum. During the experiments, attention was strictly paid to the regulations of local authorities for handling laboratory animals.

2.2. Food Deprivation

Food was withdrawn 12, 24 and 48 h prior to performing the formalin test, but water continued to be available ad libitum. Control rats had free access to both food and water.

2.3. Formalin Induced Nociceptive Behaviour

Formalin-induced nociceptive behaviour is a widely used animal model of persistent pain (Abbott, Franklin, & Westbrook, 1995; Dubuisson & Dennis, 1977). Rats (8-9 per groups) were moved to the test room at least 1 h before the commencement of the experiment. In the present study, the rats were first acclimatized for 30 minutes in an acrylic observation chamber (30 cm in diameter and in height) and then 50 µL of 2% formalin was injected subcutaneously into the plantar surface of the right hind paw with a 30 gauge needle. Each rat was then immediately returned to the observation box, and the behavioural recording commenced. A mirror, placed at a 45° angle beneath the box, permitted the observation of behaviours without moving the box. Pain behaviour was scored as follows: 0, the injected paw was not favoured; 1, the injected paw had little or no weight placed on; 2, the injected paw was elevated and not in contact with any surface; and 3, the injected paw was licked or bit. Scores were continuously observed for the duration of the experiment (90 minutes). The nociceptive behaviour score for each 3-minutes interval was calculated as the weighted average of the number of seconds spent in each nociceptive behavioural condition, from the start of the experiment. The scores were recorded in normal rats as well as in those who received 12, 24 and 48hr food deprivation. In each group, the behavioural responses of each rat during the first phase (1-7 minutes), the inter-phase (8-14), the phase 2A (15-60) and the phase 2B (61-90) were separately evaluated (Azhdari Zarmehri H. et al., 2011; Azhdari Zarmehri, Semnanian, & Fathollahi, 2008).



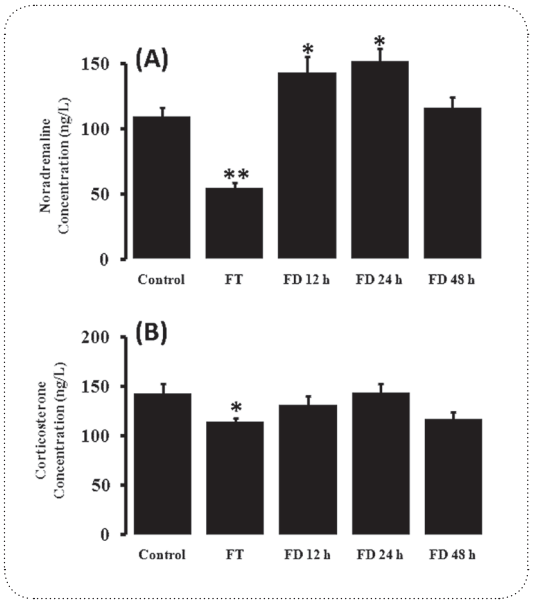


Figure 1. Effect of 12 (A), 24 (B) and 48 h (C) food deprivation on formalin test. Subcutaneous formalin (2%, 50 µL) injection into the right hind-paw of rats on the time course of the nociceptive behaviours score measured every 3 minutes for 90 minutes. The columns represent the mean of nociceptive score in each phase: phase 1 (1–7 minutes), inter-phase (8–14 minutes), phase 2A (15–60 minutes) and phase 2B (61–90 minutes).

* P<0.05; ** P<0.01; *** P<0.001 food deprivation group in comparison with control one in male rats.

2.4. Blood Sampling and Noradrenalin and Corticosterone Measurements

Under deep anaesthesia, blood was collected from the heart of rats (n=6 for each group). First from the control group and then from food deprived groups. Blood was allowed to clot and sera were separated using centrifugation at 5000 rpm for 5 min and stored at -80ºC until use. Total serum level of noradrenalin was measured using ELISA kit (Glory Science Co. USA) and total serum level of corticosterone was measured by radioimmunoassay kits (Immunotech, France). Test principle of ELISA kits was based on a double-antibody sandwich ELISA to assay noradrenalin level. In radioimmunoassay kits there is a competition between analysis in samples and 125I-labeled reagent in antibody-coated tubes.

2.5. Data Analysis

Data are presented as mean ±S.E.M. and analysed by one-way analysis of variance and t-test between groups. The mean nociceptive score in each phase (phase 1, interphase, phase 2) of the formalin test was analysed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Phase 1 (1–7 minutes), the interphase (8–14 minutes) and the phase 2 (2A: 15–60 and 2B: 61–90 minutes) of the formalin test were analysed separately. The defined level for statistical significance was P<0.05.

3. Results

3.1. Effect of 12, 24 and 48hr Food Deprivation on Formalin-Induced Nociceptive Behaviours

Food-deprived male rats were compared with non-food deprived controls to determine if the 12 h food deprivation causes the induction of significant nociceptive behaviour with formalin in rats. Following 12 and 24hr food deprivation male rats exhibited decrease in formalin induced nociceptive behaviour. There was no significant difference in behavioural response between 12hr food deprived (n=11) and control (n=11) ones during phase 1 [T(1,20)=1.688; P=0.107], while there were significant differences in the interphase [T(1,20)=3.922; P=0.001], phase 2A [T(1,20)=2.453; P=0.023] and phase 2B [T(1,20)=2.202; P=0.04]. Although 24hr food deprivation decreased nociceptive behaviour in phase 1,

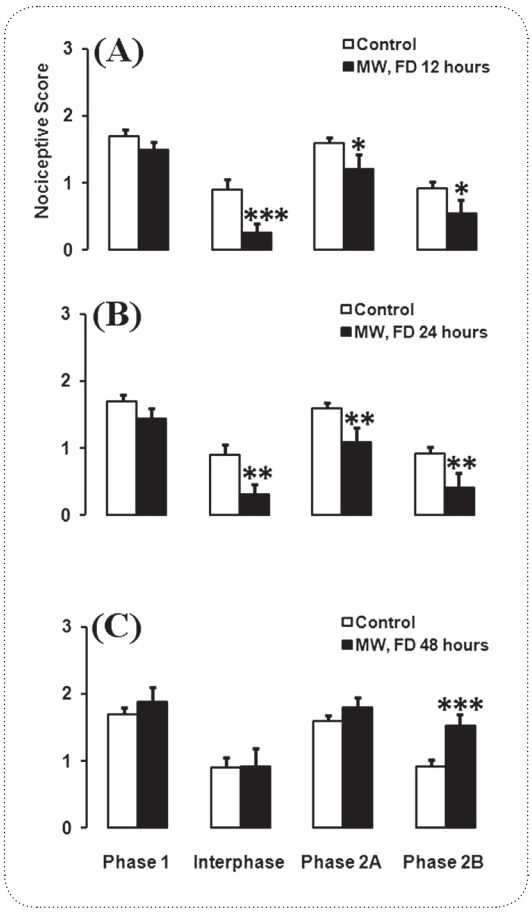
no significant difference was observed in behavioural response between 24hr food deprived (n=11) and control (n=11) ones during phase 1 [T(1,20)=1.949; P=0.065], while there were significant differences in the interphase [T(1,20)=3.572; P=0.002], phase 2A [T(1,20)=3.231; P=0.004] and phase 2B [T(1,20)=2.994; P=0.007]. Following 48hr food deprivation male rats exhibited increase in formalin induced nociceptive behaviour during phase 2. There was no significant difference in behavioural response between 48hr food deprived (n=11) and control (n=8) ones during phase 1 [T(1,16)=0.404; P=0.691], interphase [T(1,16)=0.372; P=0.714] and phase 2A [T(1,16)=1.667; P=0.114], while there were significant differences in phase 2B [T(1,16)=4.083; P=0.001; (Fig. 1.)].

3.2. Effect of Food Deprivation on Plasma Noradrenalin and Corticosterone Concentrations

Under deep anaesthesia, blood was collected from the heart of rats in order of control, formalin test (after finishing formalin test procedure), and food deprived groups (12, 24 and 48hr). The effect of food deprivation and formalin test on plasma noradrenalin level (mean ± SEM) is shown in Fig. 2. The injection of formalin in the male rats significantly decreased the concentration of plasma noradrenalin [T (1, 14) = 12.431, (p=0.003)]. Food deprivation for 12 and 24hr increased noradrenalin level in male rats, as compared with control [for 12 hours; T (1, 18)=7.990, (p=0.011), for 24 hours; T (1, 18)=3.734, (p=0.042)] and food deprivation for 48hr had no significant effect, as compared with control [T (1, 18)=0.498, (p=0.489)]. The injection of formalin in the male rats significantly decreased the concentration of plasma corticosterone [T (1, 14) = 5.430, (p=0.037)]. Food deprivation for 12, 24 and 48hr had no effect on corticosterone level in male rats, as compared with control [for 12 hours; T (1, 18)=0.276, (p=0.606), for 24 hours; T (1, 18)=0.263, (p=0.614), for 48 hours; T (1, 18)=1.372, (p=0.258)].

4. Discussion

Although 12 and 24hr food deprivation decreases nociceptive behaviour in the formalin test, 48hr food deprivation exhibited increase in formalin induced nociceptive behaviour. The injection of formalin in the male rats significantly decreased the concentration of plasma noradrenalin and corticosterone. This result is in consistent with Sajedianfard et al. (2005) who showed that after the injection of formalin, the noradrenaline concentration in the locus coeruleus increased during the first phase of the formalin test but not during the second phase. They suggested that part of the increase in noradrenaline concentration in the locus coeruleus is certainly due to the pain induced by formalin injection (Sajedianfard, Khatami, Semnanian, Naghdi, & Jorjani, 2005a). In addition, noxious stimuli, such as foot shock as well as electrical stimulation of the locus coeruleus, accelerated noradrenaline turnover in the cerebral cortex (Singewald, Kaehler, & Philippu, 1999a). Food deprivation for 12 and 24hr increased noradrenalin level in male rats which have correlated fasting induced antinociceptive behaviours and food deprivation for 48hr had no significant effect, as compared with control. Food deprivation for 12, 24 and 48hr had no effect on corticosterone level in male rats. Food deprivation and nociception are physiological conditions that are associated with homeostatic functioning and have bidirectional effect. In consistent with our result, it has been shown that food deprivation induces analgesia (McGivern, Berka, Berntson, Walker, & Sandman, 1979; Hamm, Knisely, Watson, Lyeth, & Bossut, 1985; Davidson, McKenzie, Tujo, & Bish, 1992). We demonstrated that 12 and 24hr food deprivation induced antinociceptive effect and increased noradrenaline level which might cause the analgesic effect of acute fasting. On the other hand, McGivern & Berntson in 1980 showed that this fasting induced analgesia is decreased by naloxone, suggesting that endogenous opioid systems may be involved (McGivern & Berntson, 1980). Food and/or water-deprivation induces analgesia or hyperalgesia and the magnitude of the increase in pain threshold depends on the duration of deprivation (Konecka, Sroczynska, & Przewlocki, 1985). In consistent with our study, Khasar et al. demonstrated the fasting induced pronociceptive effect (Khasar, Reichling, Green, Isenberg, & Levine, 2003). Although in both studies, formalin test was used as tonic pain model, some conditions are different between the two studies; Khasar et al. observed formalin induced nociceptive behaviour for 60 min, but in this study observation of nociceptive behaviour was followed for 90 minutes and showed pronociceptive effect during phase 2B in male rats. Other methodological differences should also be considered (e.g. fasting condition, lighting, noise, odours, handling stress or anaesthesia prior to formalin injection all known to influence the formalin test). The duration or intensity of fasting has no correlation with the level of corticosterone that is in the circulation. Although 48hr food deprivation had pronociceptive effect during the second part of phase 2, it had no significant effect on phase 1 or first part of phase 2. The difference between nociceptive behaviours in the two phases might be due to several reasons. It is thought that phase 1 may be caused by increased activity in primary afferent nociceptors due to their direct activation by formalin (Hunskaar, Berge, & Hole, 1986). Although



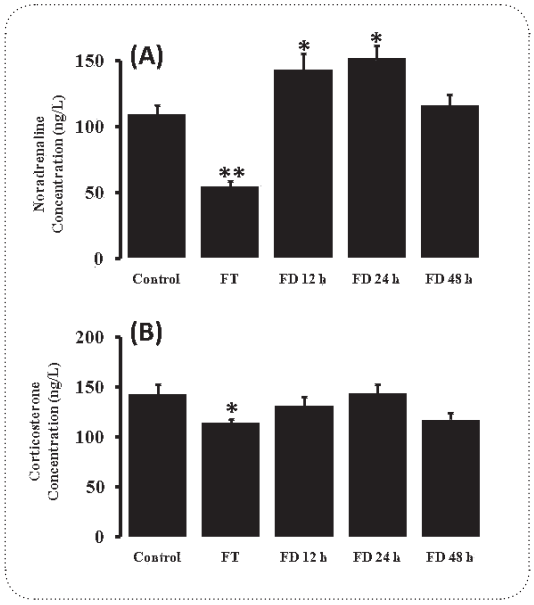


Figure 2. Effect of food deprivation on plasma noradrenalin (A) and corticosterone concentration of male rats that had ad libitum access to their diets or were food deprived for 12, 24 and 48hr.

** P<0.01 and ***P<0.01compared to control group

interphase was previously considered as an inactive phase, Henry et al (1999) showed that active inhibitory mechanisms are involved in this period (Franklin & Abbott, 1993a; Henry, Yashpal, Pitcher, & Coderre, 1999b), and it seems that sex hormones might also play a role in modulation of pain during this period (Aloisi, Albonetti, & Carli, 1994; Aloisi & Ceccarelli, 2000; Gaumond, Arsenault, & Marchand, 2002; Gaumond, Arsenault, & Marchand, 2005; Gaumond, Spooner, & Marchand, 2007). Phase 2, in addition to increased activity in sensitized primary afferent neurons, may also involve sensitization of nociceptive network in the spinal and supraspinal systems (Dickenson & Sullivan, 1987; Coderre, Vaccarino, & Melzack, 1990). Several studies suggest that the interphase of the formalin test is the result of endogenous pain-suppressing mechanisms (Franklin & Abbott, 1993b; Henry, Yashpal, Pitcher, & Coderre, 1999a). Martin et al., in 1999 showed that neurotoxic destruction of descending noradrenergic pathways had no effect on nociceptive responses in the hot-plate, tail-flick and formalin test one week post-toxin. However, two weeks post-injection, they observed a decrease in formalin-induced nociceptive behaviours in phase2 and also a reduction in formalin-evoked fos expression in the dorsal horn of spinal cord (Martin, Gupta, Loo, Rohde, & Basbaum, 1999b). They proposed that acute and persistent nociception are differentially regulated by descending noradrenergic pathways. Another study showed that electrical stimulation of neurons in the locus coeruleus induced different analgesic effect in Sprague-Dawley rats obtained from two different vendors (Harlan and Sasco rats). The analgesic effect in Harlan rats was blocked by a selective α2-adrenoceptor antagonist, yohimbine or by phentolamine, a non-selective α2-adrenoceptor antagonist, but not in Sasco rats. These observations indicate that coeruleospinal noradrenergic neurons in Harlan and Sasco Sprague-Dawley rats have different physiological functions (West, Yeomans, & Proudfit, 1993). Holden and Naleway (2001) demonstrated that neurons in the lateral hypothalamus activate spinally projecting methionine enkephalin neurons, as well as two populations of A7 noradrenergic neurons that exert a bidirectional effect on nociception (Holden & Naleway, 2001). Noradrenergic pain modulation system is affected by norepinephrine and noradrenergic receptors, supraspinal site, spinal segmental, receptor type and pain model (Pertovaara, 2006).

We suggest that extended fasting time might produce tolerance in endogenous inhibitory system that is involved in decreasing nociceptive behaviours or finishing phase 2 of formalin test. Consistent with these results, humans subjected to food deprivation without electrolyte substitute for 72hr showed an increase in plasma cortisol, beta-endorphin, noradrenalin and dopamine which were much greater on the first morning of the fasting state (Beer et al., 1989), on the contrary, the plasma noradrenalin level of the minks diminished after 3–5 days of food deprivation (Mustonen, Saarela, Pyykonen, & Nieminen, 2005c). It seems that acute fasting could increase the concentration of plasma noradrenalin which produces analgesia in formalin test. Munro in 2007 demonstrated that 5-HT and noradrenaline reuptake inhibitor decrease formalin-induced nociceptive behaviours and also showed that systemically injected dopamine receptor agonists decrease formalin-induced nociceptive behaviours in rats (Munro, 2007). Furthermore, bicifadine, a reuptake inhibitor of 5-HT, noradrenaline and dopamine has been shown to decrease nociceptive behaviours in animal pain models (Basile et al., 2007). The increase in noradrenaline after acute fasting which was observed in our study, can cause analgesic effects mediated by adrenoceptors in the dorsal horn of the spinal cord via the descending pain inhibitory pathway (Millan, 2002). For example, noradrenaline injected to the spinal cord inhibits the response of dorsal horn neurons (Millan, 2002). Inconsistent with this study, they observed brief increase in the noradrenalin concentration after 48hr of food deprivation, however, it was not significant (Mustonen, Saarela, Pyykonen, & Nieminen, 2005d). It can, however, be proposed that the increases in noradrenalin concentration is a part of the stress response following food deprivation. The decrease in the noradrenalin level after formalin test, may be associated with decreased stimulation of the sympathetic nervous system and, thus, to energy saving during food deprivation. Based on the previous studies, we suggest that increased noradrenalin concentration might induce the antinociceptive effect that happens after fasting in formalin test. We have no financial or other conflicts of interest.

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References

Abbott, F. V., Franklin, K. B., & Westbrook, R. F. (1995). The formalin test: scoring properties of the first and second phases of the pain response in rats. Pain, 60, 91-102.

Aloisi, A. M., Albonetti, M. E., & Carli, G. (1994). Sex differences in the behavioural response to persistent pain in rats. Neurosci Lett, 179, 79-82.

Aloisi, A. M. & Ceccarelli, I. (2000). Role of gonadal hormones in formalin-induced pain responses of male rats: modulation by estradiol and naloxone administration. Neuroscience, 95, 559-566.

Azhdari Zarmehri H., Semnanian, S., Fathollahi, Y., Erami, E., Khakpay, R., Azizi, H. et al. (2011). Intra-periaqueductal gray matter microinjection of orexin-A decreases formalin-induced nociceptive behaviors in adult male rats. J Pain, 12, 280-287.

Azhdari Zarmehri, H., Semnanian, S., & Fathollahi, Y. (2008). Comparing the analgesic effects of periaqueductal gray matter injection of orexin A and morphine on formalin-induced nociceptive behaviors. Physiology and Pharmacology [Article in Persian], 12, 188-193.

Basile, A. S., Janowsky, A., Golembiowska, K., Kowalska, M., Tam, E., Benveniste, M. et al. (2007). Characterization of the antinociceptive actions of bicifadine in models of acute, persistent, and chronic pain. J Pharmacol Exp Ther, 321, 1208-1225.

Beer, S. F., Bircham, P. M., Bloom, S. R., Clark, P. M., Hales, C. N., Hughes, C. M. et al. (1989). The effect of a 72-h fast on plasma levels of pituitary, adrenal, thyroid, pancreatic and gastrointestinal hormones in healthy men and women. J Endocrinol, 120, 337-350.

Bhunia, S., Bharambe, M. S., Singh, R., Premendran, J., & Pande, S. (2000). Effect of microinjections of 5-hydroxytryptamine and adrenaline in central grey on pain responsiveness during acute food deprivation in conscious rats. Indian J Exp Biol, 38, 237-241.

Bodnar, R. J., Romero, M. T., & Kramer, E. (1988). Organismic variables and pain inhibition: roles of gender and aging. Brain Res Bull, 21, 947-953.

Coderre, T. J., Vaccarino, A. L., & Melzack, R. (1990). Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection. Brain Res, 535, 155-158.

Davidson, T. L., McKenzie, B. R., Tujo, C. J., & Bish, C. K. (1992). Development of tolerance to endogenous opiates activated by 24-h food deprivation. Appetite, 19, 1-13.

Dickenson, A. H. & Sullivan, A. F. (1987). Peripheral origins and central modulation of subcutaneous formalin-induced activity of rat dorsal horn neurones. Neurosci Lett, 83, 207-211.

Dubuisson, D. & Dennis, S. G. (1977). The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain, 4, 161-174.

Franklin, K. B. & Abbott, F. V. (1993b). Pentobarbital, diazepam, and ethanol abolish the interphase diminution of pain in the formalin test: evidence for pain modulation by GABAA receptors. Pharmacol Biochem Behav, 46, 661-666.

Franklin, K. B. & Abbott, F. V. (1993a). Pentobarbital, diazepam, and ethanol abolish the interphase diminution of pain in the formalin test: evidence for pain modulation by GABAA receptors. Pharmacol Biochem Behav, 46, 661-666.

Gaumond, I., Arsenault, P., & Marchand, S. (2002). The role of sex hormones on formalin-induced nociceptive responses. Brain Res,958, 139-145.

Gaumond, I., Arsenault, P., & Marchand, S. (2005). Specificity of female and male sex hormones on excitatory and inhibitory phases of formalin-induced nociceptive responses. Brain Res, 1052, 105-111.

Gaumond, I., Spooner, M. F., & Marchand, S. (2007). Sex differences in opioid-mediated pain inhibitory mechanisms during the interphase in the formalin test. Neuroscience, 146, 366-374.

Hamm, R. J. & Knisely, J. S. (1986). The analgesia produced by food deprivation in 4-month old, 14-month old, and 24-month old rats. Life Sci, 39, 1509-1515.

Hamm, R. J., Knisely, J. S., Watson, A., Lyeth, B. G., & Bossut, D. F. (1985). Hormonal mediation of the analgesia produced by food deprivation. Physiol Behav, 35, 879-882.

Henry, J. L., Yashpal, K., Pitcher, G. M., & Coderre, T. J. (1999b). Physiological evidence that the 'interphase' in the formalin test is due to active inhibition. Pain, 82, 57-63.

Hentall, I. D., Mesigil, R., Pinzon, A., & Noga, B. R. (2003). Temporal and spatial profiles of pontine-evoked monoamine release in the rat's spinal cord. J Neurophysiol, 89, 2943-2951.

Holden, J. E. & Naleway, E. (2001). Microinjection of carbachol in the lateral hypothalamus produces opposing actions on nociception mediated by alpha(1)- and alpha(2)-adrenoceptors. Brain Res, 911, 27-36.

Hunskaar, S., Berge, O. G., & Hole, K. (1986). Dissociation between antinociceptive and anti-inflammatory effects of acetylsalicylic acid and indomethacin in the formalin test. Pain, 25, 125-132.

Jones, S. L. (1991). Descending noradrenergic influences on pain. Prog Brain Res, 88:381-94., 381-394.

Khasar, S. G., Reichling, D. B., Green, P. G., Isenberg, W. M., & Levine, J. D. (2003). Fasting is a physiological stimulus of vagus-mediated enhancement of nociception in the female rat. Neuroscience, 119, 215-221.

Konecka, A. M., Sroczynska, I., & Przewlocki, R. (1985). The effect of food and water deprivation on post-stress analgesia in mice and levels of beta-endorphin and dynorphin in blood plasma and hypothalamus. Arch Int Physiol Biochim, 93, 279-284.

Kwiat, G. C. & Basbaum, A. I. (1992). The origin of brainstem noradrenergic and serotonergic projections to the spinal cord dorsal horn in the rat. Somatosens Mot Res, 9, 157-173.

Martin, W. J., Gupta, N. K., Loo, C. M., Rohde, D. S., & Basbaum, A. I. (1999b). Differential effects of neurotoxic destruction of descending noradrenergic pathways on acute and persistent nociceptive processing. Pain, 80, 57-65.

McGivern, R., Berka, C., Berntson, G. G., Walker, J. M., & Sandman, C. A. (1979). Effect of naloxone on analgesia induced by food deprivation. Life Sci, 25, 885-888.

McGivern, R. F. & Berntson, G. G. (1980). Mediation of diurnal fluctuations in pain sensitivity in the rat by food intake patterns: reversal by naloxone. Science, 210, 210-211.

Millan, M. J. (2002). Descending control of pain. Prog Neurobiol, 66, 355-474.

Munro, G. (2007). Dopamine D1 and D2 receptor agonism enhances antinociception mediated by the serotonin and noradrenaline reuptake inhibitor duloxetine in the rat formalin test. European Journal of Pharmacology, 575, 66-74.

Mustonen, A. M., Saarela, S., Pyykonen, T., & Nieminen, P. (2005b). Endocrinologic adaptations to wintertime fasting in the male American mink (Mustela vison). Exp Biol Med (Maywood), 230, 612-620.

Nalepa, I., Vetulani, J., Borghi, V., Kowalska, M., Przewlocka, B., & Pavone, F. (2005). Formalin hindpaw injection induces changes in the [3H]prazosin binding to alpha1-adrenoceptors in specific regions of the mouse brain and spinal cord. J Neural Transm, 112, 1309-1319.

Pertovaara, A. (2006). Noradrenergic pain modulation. Prog Neurobiol, 80, 53-83.

Proudfit, H. K. (1988). Pharmacologic evidence for the modulation of nociception by noradrenergic neurons. Prog Brain Res, 77:357-70., 357-370.

Safari, M. S., Haghparast, A., & Semnanian, S. (2009). Effect of lidocaine administration at the nucleus locus coeruleus level on lateral hypothalamus-induced antinociception in the rat. Pharmacology Biochemistry and Behavior, 92, 629-634.

Sajedianfard, J., Khatami, S., Semnanian, S., Naghdi, N., & Jorjani, M. (2005b). In vivo measurement of noradrenaline in the locus coeruleus of rats during the formalin test: A microdialysis study. European Journal of Pharmacology, 512, 153-156.

Sajedianfard, J., Khatami, S., Semnanian, S., Naghdi, N., & Jorjani, M. (2005a). In vivo measurement of noradrenaline in the locus coeruleus of rats during the formalin test: A microdialysis study. European Journal of Pharmacology, 512, 153-156.

Singewald, N., Kaehler, S. T., & Philippu, A. (1999b). Noradrenaline release in the locus coeruleus of conscious rats is triggered by drugs, stress and blood pressure changes. Neuroreport, 10, 1583-1587.

Takagi, H., Shiomi, H., Kuraishi, Y., Fukui, K., & Ueda, H. (1979). Pain and the bulbospinal noradrenergic system: pain-induced increase in normetanephrine content in the spinal cord and its modification by morphine. Eur J Pharmacol, 54, 99-107.

Tyce, G. M. & Yaksh, T. L. (1981). Monoamine release from cat spinal cord by somatic stimuli: an intrinsic modulatory system. J Physiol, 314:513-29., 513-529.

Uvnas-Wallenstein, K. & Palmblad, J. (1980). Effect of food deprivation (fasting) on plasma gastrin levels in man. Scand J Gastroenterol, 15, 187-191.

West, W. L., Yeomans, D. C., & Proudfit, H. K. (1993). The function of noradrenergic neurons in mediating antinociception induced by electrical stimulation of the locus coeruleus in two different sources of Sprague-Dawley rats. Brain Res, 626, 127-135.

Yaksh, T. L. & Tyce, G. M. (1981). Release of norepinephrine and serotonin in cat spinal cord: direct in vivo evidence for the activation of descending monoamine pathways by somatic stimulation. J Physiol (Paris), 77, 483-487.

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Senden, T. J., Moock, K. H., Gerald, J. F., Burch, W. M., Bowitt, R. J., & Ling, C. D., et al. (1997). The physical and chemical nature of technigas. Journal of Nuclear Medicine, 38(10), 1327-1333.

Sanchez, D., & King-Toler, E. (2007). Addressing disparities consultation and outreach strategies for university settings. Consulting Psychology Journal: Practice and Research, 59(4), 286-295. doi:10.1037/1065-9293.59.4.286.

Reference to a book:

Frank, R. H., & Bernanke, B. (2007). Principles of macro-economics (3rd ed.). Boston: McGraw-Hill/Irwin.

Flower, L., Hayes, J. R., Carey, L., Schriver, K., & Stratman, J. (1986). Detection, diagnosis, and strategies of revision. In L. Ede (Ed.), The Braddock essays (pp. 191-228). Boston: Bedford St. Martin's.

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