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Creatine Monohydrate Supplemented Diets Affect Serum Inflammatory Cytokine Levels (IL-6 and IL-18) in Female Albino Mice Following Neonatal Hypoxic Ischemic Encephalopathy

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Abstract.- Inflammatory mediators are produced after hypoxia ischemia (HI) in the immature brain and plays a critical role in the pathogenesis of brain injury. Interleukin 6 (IL-6) and 18 (IL-18) are proinflammatory cytokines involved in the pathogenesis of HI induced brain damage. The present study was designed to demonstrate the influence of 1% and 3% creatine monohydrate (Cr) supplemented diet on concentration of IL (6 and 18) in female albino mice following neonatal brain damage. Female pups on postnatal day (PN) 10 were subjected to hypoxic ischemic brain injury (HI) by unilateral (left) carotid ligation and exposure to 8% oxygen for 25 minutes. Following weaning on PN 20 mice were fed with 1% and 3% Cr supplemented diet for a period of 10 weeks. A control group was maintained in parallel with similar experimental conditions but supplemented with normal rodent diet. The interleukin-6 (IL-6) and interleukin-18 (IL-18) concentrations were measured in the serum by Enzyme linked immunosorbent assay. Our results revealed increased serum IL-6 and and decreased IL-18 concentrations in 3% Cr supplemented mice indicating that Cr has a potential to mediate the levels of both cytokines and can play a protective role in neonatal brains exposed to HI insult.

Keywords: Hypoxic ischemic encephalopathy (HIE), creatine monohydrate, interleukin-6 (IL-6), interleukin-18 (IL-18), enzyme linked immunosorbent assay (ELISA).

INTRODUCTION

A cascade of pathophysiological processes initiates following hypoxia ischemia (HI) which contributes to neuronal damage. Inflammation is a critical factor in this pathway of ischemic cell damage which involves the production of inflammatory mediators (cytokines) in the immature brain (Sun *et al*., 2005; del Zoppo *et al*., 2000; Bona *et al*., 1999). The presence of infiltrating leukocytes resulting in inflammation to the ischemic area (as with other inflammatory diseases) postulates a role for cytokines in this process (Pantoni *et al*., 1998).

Interleukins are a group of cytokines that act specifically as modulators of inflammatory response. The function of the immune system depends, in a large part, on interleukins. The brain exhibits adaptive immunity in response to an injury and inflammatory mediators, *i.e*., cytokines, released during an innate immune response (Sun *et al*., 2005). Recent clinical and experimental evidence indicates that some cytokines strongly influence neurons and their ability to process information making inflammation a leading factor in the pathogenesis of neonatal hypoxic–ischemic brain injury (Chen *et al*., 2007; Grow and Barks, 2002; Bona *et al*., 1999; Hagberg *et al*., 1996). The studied interleukins; IL-6 and IL-18 are pleiotropic proinflammatory cytokines involved in the inflammatory cascade (Chen *et al*., 2007; Gracie *et al*., 2003). IL-6 promotes neuroprotection in HI induced injury (Gadient and Otten, 1997) while IL-18 participates in neuroinflammatory/ neurodegenerative processes and also influences homeostasis and behaviour (Alboni *et al*., 2010).

Creatine (Cr) is a nutritional supplement and it provides sufficient ATP (adenosine triphosphate) concentrations that maintain physiological processes and has been reported to protect brain tissues from hypoxic damage (Berger *et al*., 2004; Wilkena *et al*., 2000). Oral Cr supplementation has been demonstrated to be neuroprotective in a variety of neurological disease models and *in-vitro* and *in-vivo* animal studies (rat, mouse) and to protect the developing brain against the long-term deleterious effects of HI insult (Zhu *et al*., 2004; Berger *et al*.,

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2004; Adcock *et al*., 2002; Brustovetsky *et al*., 2001; Wilkena *et al*., 2000). A lot of research has been carried out on creatine and interleukin role in and/or after HIE but this is for the first time that a relationship between interleukin levels in blood sera and administration of different concentrations of Cr, as a treatment for HI, has been established. The present study was designed to determine the influence of two different doses (1% and 3%) of creatine monohydrate on serum levels of proinflammatory cytokines (IL-6 and IL-18) in female albino mice with neonatal HI insult.

MATERIALS AND METHODS

Female albino mice were used as experimental subjects. Mouse breeding pairs were supplied by Veterinary Research Institute, Ghazi Road, Lahore, Pakistan. All animals were housed at Neuroscience Research Lab at core animal facility of Bahauddin Zakariya University, Multan; where a breeding colony was established to generate mouse littermates to be used in this experimental study. These mice were kept in cages at room temperature (22±1°C). Cages were filled with wood chips and cotton, with free access to diet and water. The room was illuminated with artificial light to maintain light/dark rhythm of 14:10 h. All mouse handling techniques and experimental procedure were approved by the ethical committee of Institute of Pure and Applied Biology (IPAB), Bahauddin Zakariya University, Multan, Pakistan.

On, postnatal day 10, corresponding to the brain development of full-term human infant of 40 weeks gestational age (Romijn *et al*., 1991), pups were subjected to isoflurane (for induction, 3-4%; for maintenance, 1.5-2.5%) anesthesia. A small lateral left neck incision was made and the left common carotid artery was permanently ligated by using polypropylenedalcon USP 6 suture (Ethicon, USA). After a half hour recovery in dams pups were exposed to hypoxia in a controlled atmosphere chamber that was infused with 8% oxygen balanced with nitrogen for 25 min. Pups were placed on a hot plate during whole surgical and hypoxic procedure with constant 36°C temperature. An oxygen analyzer (BW, Canada) was used to monitor the oxygen concentration inside the chamber. Pups were returned to their dams until weaning. After weaning on PN 20, mice were placed in individual cages. Control group received normal rodent diet while experimental treatments received 1% and 3% Cr supplemented diet (Ssniff, Germany) for 10 weeks.

At the end of experimental duration, blood sampling was performed from retro-orbital sinus under chloroform inhalation. Blood was centrifuged for ten minutes at 13000 rpm and plasma was separated for biochemical analysis of cytokines (IL-6 and IL-18) through enzyme-linked immunoabsorbent assay, ELISA, by using the diagnostic kits [MBL, for interleukin-18 and Invitrogen, CMC0063 for interleukin 6] following manufacturer's recommendations.

One way ANOVA test was applied to determine the effect of creatine supplementation treatment on IL levels. Analysis was carried out using statistical package Minitab 16 (USA). All the data is presented as Mean ± Standard deviation.

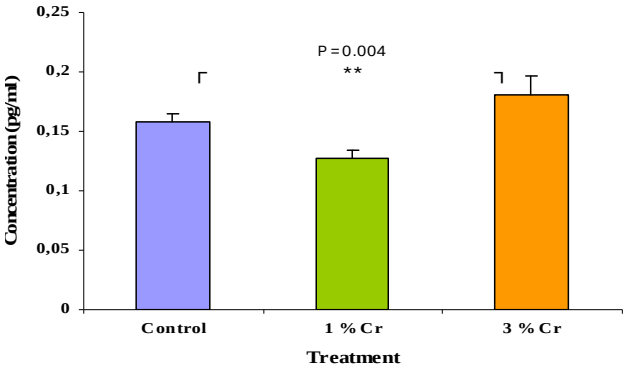
RESULTS

Determination of interleukin 6 (IL-6) in serum samples from the three experimental treatments indicated a significant affect of diet on studied parameter ($P = 0.004$). Data obtained revealed that 3% Cr supplemented group had highest plasma IL6 concentration indicating the up regulation of inflammatory response following hypoxic ischemic insult (Fig. 1).

Comparison of interleukin 18 (IL-18) in serum revealed a trend of decreasing IL-18 concentrations with an increase of creatine monohydrate in diet but this difference in IL-18 concentration between the treatments was statistically non significant ($P = 0.16$) (Fig. 2).

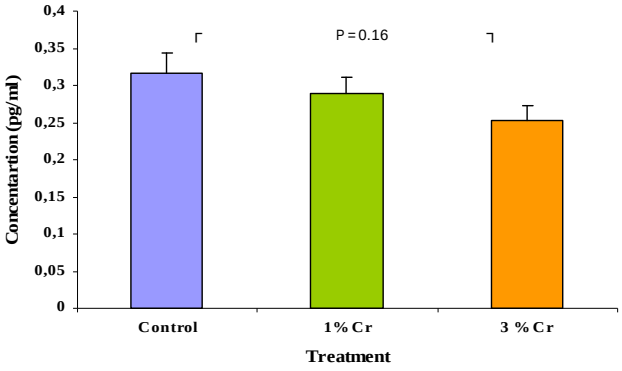
DISCUSSION

Inflammation is a critical factor in the development of HI mediated brain injury and interleukins plays the major role in mediating inflammation (Clarkson *et al*., 2005; Hedtjarn *et al*., 2002; Bona *et al*., 1999). Interleukin-6 (IL-6) is confirmed proinflammatory cytokine which plays central role in the inflammatory response following



P < 0.01 = Significant (**)

Fig. 1. Comparison of Interleukin 6 concentrations in serum of female albino mice with or without dietary Creatine monohydrate supplementations for 10 weeks following neonatal hypoxic ischemic insult.
P-values indicate one way ANOVA results.



P > 0.05 = Non significant

Fig. 2. Comparison of Interleukin 18 concentrations in serum of female albino mice with or without dietary Creatine monohydrate supplementations for 10 weeks following neonatal hypoxic ischemic insult. One way ANOVA revealed no significant affect of diet supplementation.

cerebral hypoxia ischemia (Loddick *et al*., 1998). It has been reported that IL-6 is up-regulated in the immature brain after HI (Chen *et al*., 2007; Feng and LeBlanc, 2003; Hedtjarn *et al*., 2002; Peeters-Scholte *et al*., 2002). As hypoxia is a significant threat to neuronal cell function and survival thus, it is possible that release of IL-6 serves as an adaptive function in this context, giving its neuroprotective properties (Hirota *et al*., 1996; Kushima *et al*., 1992). It has also been documented that IL-6 prevents ischemia-induced neuronal cell loss (Loddick *et al*., 1998) and cognitive deficits (Matsuda *et al*., 1996) when administered *in vivo*. Analysis of the results indicated that mice supplemented with 3% Cr following neonatal brain damage had higher level of IL-6 in serum as compared to mice on normal rodent diet (Fig. 1). This observation supports our finding that Cr treated mice showed much better performance as compared to control ones (mouse on normal rodent diet) indicating that Cr supplementation may rescue the neurons exposed to hypoxic ischemic insult by stimulating the energy metabolism. We have recently reported the effect of creatine monohydrate supplementation on complete blood count and on selected parameters of serum biochemical profile in albino mice following hypoxic ischemic insult and we had observed that creatine supplementation following hypoxic ischemic insult helps in maintaining the normal blood chemistry when compared with the creatine untreated mice indicating that creatine reaches the target sites in intact form when transported via blood following dietary supplementation for variable durations (Iqbal *et al*., 2013).

Previous studies report morphological microglial activation in the brain after physical/ emotional stress and this activation is in part mediated by interleukin 18 (IL-18) (Sugama *et al*., 2007). In present study, IL-18 showed a decreasing trend (Fig. 2) with increasing Cr concentration in diet and we have also observed that increasing Cr concentration in diet is correlated with better neurological performance after brain injury indicating that decreasing IL-18 concentrations are also associated with Cr mediated neuroprotection in female albino mice (Data not shown here). Chen *et al*. (2007) had reported an increased IL-18 concentration after HI brain injury than in control groups. On the other hand Hedtjarn *et al*. (2002) has reported that IL-18 deficient mice are protected from HI indicating its negative role after HI brain injury. They had also reported that IL-18 knockout mice had a 21% decrease in infarct size after HI as compared to wild-type mice. The present study reports a decreased level of interleukin 18 after Cr treatment providing evidence that Cr

supplementation may lead to neuroprotection by decreasing the serum IL-18 concentrations.

It is concluded from our study that both cytokines are are playing some significant roles in albino mice following HI mediated brain damage. As Concentration of IL-6 was higher in Cr supplemented treatment while the concentration of IL-18 was lower indicating that Cr is regulating the IL-6 and 18 depended cascades in order to neutralize the brain injury as IL-6 is known to be neuroprotective and IL-18 as neurodegenerative cytokine.

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Conflict of interest

Authors have no conflict of interest of any sort with any one.

REFERENCES

ADCOCK, K. H., NEDELCU, J., LOENNEKER, T., MARTIN, E., WALLIMANN, T. AND WAGNER, B. P., 2002. Neuroprotection of creatine supplementation in neonatal rats with transient cerebral hypoxia-ischemia. *Dev. Neurosci*., **24**: 382-388.

ALBONI, S., CERVIA, D., SUGAMA, S. AND CONTI, B., 2010. Interleukin 18 in the CNS. *J. Neuroinflam.*, **7**: 9-21.

BERGER, R., MIDDELANIS, J., VAIHINGER, H. M., MIES, G., WILKEN, B. AND JENSEN, A., 2004. Creatine protects the immature brain from hypoxic-ischemic injury. *J. Soc. Gynecol. Invest.*, **11**: 9-15.

BONA, E. ANDERSON, A. L., BLOMGREN, K., GILLAND, E., PUKA-SUNDVALL, M., GUSTAFSON, K. AND HAGBERG, H., 1999. Chemokine and inflammatory cell response to hypoxia-ischemia in immature rats. *Pediat. Res.*, **45**: 500-509.

BRUSTOVETSKY, N., BRUSTOVETSKY, T. AND DUBINSKY, J. M., 2001. On the mechanisms of neuroprotection by creatine and phosphocreatine. *J. Neurochem.*, **76**: 425-34.

CHEN, J., HUANG, H., ZHENG, K., XIA, R., CHEN, J., YANG, X., YE, C. AND LIN, Q., 2007. Early application of nerve growth factor affects serum inflammatory cytokine levels in neonatal hypoxic ischemic encephalopathy. *Neural Regen. Res.*, **2**: 437-439.

CLARKSON, A. N., BRAD, A. N., SUTHERLAND, A. AND APPLETON, I., 2005. The biology and pathology of hypoxia-ischemia: an update. *Arch. Immunol. Ther. Exp*., **53**: 213-225.

DEL ZOPPO, G., GINIS, I., HALLENBECK, J. M., IADECOLA, C., WANG, X. AND FEUERSTEIN, G. Z., 2000. Inflammation and stroke: putative role for cytokines, adhesion molecules and iNOS in brain response to ischemia. *Brain Pathol*., **10**: 95-112.

FENG, Y. AND LE BLANC, M. H., 2003. Effect of agmatine on the time course of brain inflammatory cytokines after injury in rat pups. *Ann. NY Acad. Sci*., **1009**: 152-156.

GADIENT, R. A. AND OTTEN, U. H., 1997. Interleukin-6 (IL-6), a molecule with both beneficial and destructive potentials. *Prog. Neurobiol*., **52**: 379-390.

GRACIE, J. A., SUSAN, E. R. AND MCLNNES, B., 2003. Interleukin-18. *J. Leukocyte Biol*., **73**: 213-224.

GROW, J. AND BARKS, J. D., 2002. Pathogenesis of hypoxic-ischemic cerebral injury in the term infant: current concepts. *Clin. Perinatol*., **29**: 585-602.

HAGBERG, H., GILLAND, E., BONA, E., HANSON, L. A., HAHN-ZORIC, M., BLENNOW, M., HOLST, M., MCRAE, A. AND SODER, O., 1996. Enhanced expression of interleukin (IL)-1 and IL-6 messenger RNA and bioactive protein after hypoxia-ischemia in neonatal rats. *Pediat. Res.*, **40**: 603-609.

HEDTJARN, M., LEVERIN, A.L., ERIKSSON, K., BLOMGREN, K., MALLARD, C. AND HAGBERG, H., 2002. Interleukin-18 involvement in hypoxic-ischemic brain injury. *J. Neurosci*., **22**: 5910-5919.

HIROTA, H., KIYAMA, H., KISHIMOTO, T. AND TAGA, T., 1996. Accelerated nerve regeneration in mice by upregulated expression of interleukin IL-6 and IL-6 receptor after trauma. *J. exp. Med*., **183**: 2627-2634.

IQBAL, S., NAZIR, N., GILLANI, Q. U. A., AKBAR, A. AND IQBAL, F., 2013. Effect of creatine monohydrate supplementation on various hematological and serum biochemical parameters of male albino mice following neonatal hypoxia-ischemia encephalopathy. *Sci. World J*., http://dx.doi.org/10.1155/2013/286075.

KUSHIMA, Y., HAMA, T. AND HATANAKA, H., 1992. Interleukin-6 as a neurotrophic factor for promoting the survival of cultured catecholaminergic neurons in a chemically defined medium from fetal and postnatal rat midbrains. *Neurosci. Res.*, **13**: 267-281.

LODDICK, S. A., TURNBULL, A. V. AND ROTHWELL, N. J., 1998. Cerebral interleukin-6 is neuroprotective during permanent focal cerebral ischemia in the rat. *J. Cereb. Blood Flow Metabol*., **18**: 176-179.

MATSUDA, S., WEN, T. C., MORITA, F., OTSUKA, H., IGASE, K., YOSHIMURA, H. AND SAKANAKA, M., 1996. Interleukin-6 prevents ischemia-induced learning disability and neuronal and synaptic loss in gerbils. *Neurosci. Lett*., **204**: 109-112.

PANTONI, L., SARTI, C. AND INZITARI, D., 1998. Cytokines and cell adhesion molecules in cerebral ischemia: experimental bases and therapeutic perspectives. *Arterioscler. Thromb. Vasc. Biol*., **18**: 503-513.

PEETERS-SCHOLTE, C., KOSTER, J., VAN DEN TWEEL, E., BLOMGREN, K., HAMERS, N., ZHU, C., VAN BUUL-OFFERS, S., HAGBERG, H., VAN BEL, F., HEIJNEN, C. AND GROENENDAAL, F., 2002. Effects of selective nitric oxide synthase inhibition on IGF-1, caspases and cytokines in a newborn piglet model of perinatal hypoxia-ischaemia. *Dev. Neurosci.*, **24**: 396-404.

ROMIJN, H. J., HOFMAN, M. A. AND GRAMSBERGEN, A., 1991. At what age is the developing cerebral cortex of the rat comparable to that of the full-term newborn human baby? *Early Hum. Devel.*, **26**: 61-67.

SUGAMA, S., FUJITA, M., HASHIMOTO, M. and CONTI, B., 2007. Stress induced morphological microglial activation in the rodent brain: involvement of interleukin-18. *Neuroscience*, **146**: 1388-1399.

SUN, Y., CALVERT, J. W. AND ZHANG, J. H., 2005. Neonatal hypoxia/ischemia is associated with decreased inflammatory mediators after erythropoietin administration. *J. Am. Heart Assoc.*, **36**: 1672-1678.

WILKENA, B., RAMIREZ, J. M., PROBST, I., RICHTER, D. W. AND HANEFELD, F., 2000. Anoxic ATP depletion in neonatal mice brainstem is prevented by creatine supplementation. *Arch. Dis. Child. Fetal Neonatal Ed*., **82**: 224-227.

ZHU, S., LI, M., FIGUEROA, B. E., LIU, A., STAVROVSKAYA, I. G., PASINELLI, P., BEAL, M. F., ROBERT, H., KRISTAL, B. S., ROBERT, J., FERRANTE, R. J. AND FRIEDLANDER, R. M., 2004. Prophylactic creatine administration mediates neuroprotection in cerebral ischemia in mice. *J. Neurosci*., **24**: 5909-5912.

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