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AIF, PARP-1 and MicroRNA-9 Expression in Cerebral Ischemia Associated to Alcoholism Experimental Model

Expresión de AIF, PARP-1 y MicroRNA-9 de Isquemia Cerebral Asociado a un Modelo Experimental de Alcoholismo

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SUMMARY: The chronic consumption of alcohol causes a worsening of the events that follow the cerebral ischemia. These events are regulated through the expression of several genes and microRNAs. The aimof this work was To analyze and describe the expression profile of PARP and AIF and miRNA-9 proteins in rats submitted to focal cerebral ischemia, associated or not with chronic alcoholism model. Methods: Twenty adult Wistar rats, subdivided into: control; ischemic; alcoholic and ischemic / alcoholized for immunohistochemical analysis and miRNA-9 gene expression. Results: There was a reduction in the protein expression of PARP-1 and a positive marking for AIF in the ischemic / alcoholized group. The miRNA-9 did not obtain significant expression. The association of ischemia with chronic alcohol use promoted a tendency to low expression of miRNA-9, low expression of PARP-1 and high expression of AIF, indicating an interference in the protective effect of miRNA-9 be observed in the other groups.

KEY WORDS: PARP-1; AIF; miRNA-9; Cerebral ischemia; Alcoholism.

INTRODUCTION

Cerebral ischemia is one of the most serious cerebrovascular diseases, causing morbidity and mortality worldwide (Donnan *et al*., 2008; Mandel *et al*., 2012).

The use of alcoholic beverages is a risk factor for cerebrovascular accident and cardiovascular diseases (O'Keefe *et al*., 2014).

In addition to advanced intravascular procedures, the most commonly used treatment for cerebral ischemia is the administration of thrombolytic agents, recombinant tissue plasminogen activators and calcium ion antagonists (Ginsberg, 2008). However, some challenges such as the early administration of these drugs, as well as their frequent side effects, may be an obstacle to clinical efficacy (Goldstein & Rothwel, 2012).

Ischemic stroke is responsible for a large proportion of cerebrovascular disease. The main pathophysiological process related to brain injury is apoptosis (Abas *et al*., 2010; Wang *et al*., 2017).

Apoptotic cells are present in the ischemic area and this can be reduced with adequate pharmacological treatment (Sobrado *et al*., 2003). The duration and severity of the ischemic process determine whether the cells will undergo rapid death by necrosis or slower and more controlled deterioration, culminating in death by apoptosis (Mehta *et al*., 2007).

PARP-1 / AIF. The study of poly (ADP-ribose) polymer (PAR) occurs because of its influence on mitosis progression, DNA repair mechanisms, transcriptional control and caspase-independent cell death. Traditionally, PAR is produced in response to DNA damage, but its role in cell death suggests that alterations to the synthesis or metabolism of PAR may be interesting therapeutic options for a variety of diseases (Heeres & Hergenrother, 2007).

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The major enzyme responsible for the production of PAR polymers is poly (ADP-ribose) polymerase-1 (PARP-1), corresponding to more than 99 % of the synthesis of PAR in the cell during genotoxic stress (Sallmann *et al*., 2000; Hassa *et al*., 2006).

PARP-1 is an abundant nuclear protein that is involved in the repair of excision of the DNA base system, which is potently activated by notches and breaks in the DNA chain (Yu *et al*., 2006).

Although the exact physiological function of PARP-1 is not fully understood, in some tissues it plays an important role in DNA repair and genomic stability. Catabolism and metabolism of Poly (ADP-ribose) (PAR) is a dynamic process, with PAR glycohydrolase (PARG) playing an important role in polymer degradation (Lin *et al*., 1997).

Excessive activation of PARP-1 leads to an intrinsic and unique cell death program where the PAR polymer participates in PARP-1 dependent cell death. Measures that interfere with PAR actions may offer innovative therapeutic approaches to treat cell injury (Gibson *et al*., 2008).

AIF is also associated with the death of nerve cells. Excessive activation of nuclear ADP-ribose poly (PARP) sends the signal to the mitochondria, causing release of AIF from the mitochondria to the cell nucleus causing DNA fragmentation and chromatin condensation (Lee *et al*., 2004).

AIF has two independent functions: one within the mitochondria, involved in cell survival, probably by assembly or stabilization of the respiratory complex I and another as a promoter of cell death, due to the activation of PARP- 1, which releases AIF to the cytoplasm that will enter the nucleus to induce cell death (David *et al*., 2009).

This pathway of cellular suicide has been implicated in several clinical situations, such as stroke, myocardial ischemia, diabetes, cardiovascular dysfunction associated with diabetes, shock and central nervous system injuries due to trauma, etc. (Sriram *et al*., 2015).

MATERIAL AND METHOD

For this study, 40 male Wistar rats weighing approximately 280 to 310 grams were used. The animals were kept under standard conditions, with light cycle of 12 hours, under controlled temperature and relative humidity, in appropriate cages and submitted to standard feed and water ad libitum until euthanasia.

The design of the experiments carried out in this study was submitted and approved by the Ethics Committee on Animal Experimentation (CETEA) of Medicine School of Ribeirão Preto – University of São Paulo.

Experimental groups: The animals were randomly divided into 4 groups: group C (control); Group I (ischemic), where the animals were subjected to focal ischemia by ACM occlusion for 90 minutes, followed by reperfusion of 48 hours; group A (alcoholic), where the animals received ethanol diluted to 20 % in water for four weeks and were then sacrificed; group IA (ischemic and alcoholic), where the animals were submitted to the same alcoholic protocol of group A and the same group I ischemia protocol.

Alcoholic Groups Protocol: The animals of groups A and IA were submitted to the model of "semi-voluntary alcoholism". As the only source of liquid available to the animals, ethanol diluted in water. For alcoholic habituation, the initial dilution concentration is 5 % for one week, gradually increasing to 10 % in the second week and to 20 % in the third week. After the third week, the surgical procedure for cerebral ischemia performed.

Surgical procedure: The surgical procedure for the performance of ischemia consists of the occlusion of the middle cerebral artery (ACM) by the retrograde introduction of an obstructing wire Carlotti Junior *et al*. (2001). The animals were maintained under stable conditions during the period of ischemia and after the 90 minutes period of focal ischemia had elapsed. The reperfusion period was 48 hours.

Immunohistochemical analysis: After the reperfusion period, euthanasia was performed and sections of the brain tissue were submitted to immunohistochemical analysis for antibodies related to the apoptosis mechanism: PARP 1 and AIF.

The expression of the proteins studied was performed under a microscope (Axioskop 2 plus, Zeiss, Germany), increasing by 400x. Two parameters were analyzed:

A) Percentage of positively labeled cells (neurons and cells of neuroglia) in dorsolateral cortex (CDL) and area lateral cortex (CL), both of the left cerebral hemisphere. For counting, two fields with a magnification of 400X, for each area under study were chosen in the regions of the cortex (layers): a medial field and a lateral field. However, in each field chosen, the counting was performed on the layer (s) where there was the highest positive mark (hot spot regions) for the respective protein under study (PARP 1 and AIF). From the count of the total number of positive and negative cells, the percentage of positive cells was calculated.

B) Percentage of positively labeled cells (neurons and neuroglia cells) in the striatum (S) of the left cerebral hemisphere. For the cell counts, two fields were chosen in a 400X magnification: a medial field and a lateral field (Fig. 2). However, in each field chosen, the counting was performed in the region where there was the highest positive cell marking (hot spot regions) for the respective protein under study (PARP 1 and AIF). From the count of the total number of positive and negative cells, the percentage of positive cells was calculated.

Analysis of the serum gene expression of MiRNA-9: For the analysis of gene expression, 1 ml of blood was collected in a single moment for the four groups under study using the real-time PCR method with the use of TaqMan Master Mix reagent (Applied Biosystems).

The data were constantly collected during the PCR and analyzed in ABI-7500 SDS software package.

Statistical analysis: The Kruskal-Wallis test and the Dunn´s multiple-comparison test of the GraphPad Prism version 4.00 for Windows program (GraphPad Software, San Diego - California USA) were used for the evaluation of protein expression and gene expression. Statistically significant values of $p < 0.05$.

RESULTS

Immunohistochemical analysis of PARP-1/AIF: It was noted positive nuclear stain for PARP-1 protein in all the groups analyzed at areas CDL, CL and S. The comparison among the groups identified a decrease at PARP-1 protein expression in animals from group AI when compared to the others (Figs. 1 A, B, C).

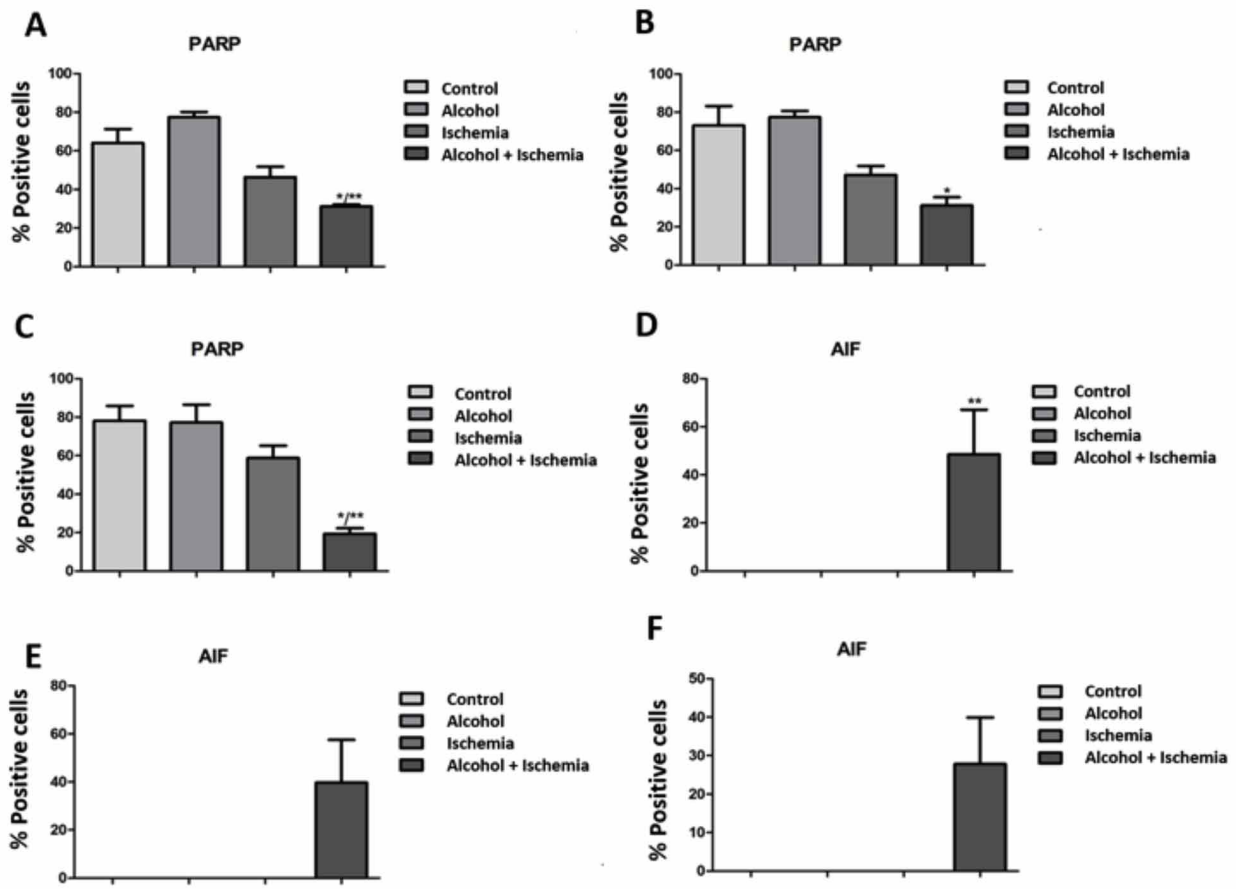

Fig. 1. Graph representation of average and standard variation of: A- PARP expression at CDL at analyzed groups (p=0.0014 Kruskal-Wallis test). Group C versus AI (p=0.0079**); group A versus AI (p=0.0119*), Dunn´s post test. B- PARP expression at CL at analyzed groups (p=0.0045 Kruskal-Wallis test). Group C versus AI (p=0.0159**); group A versus AI (p=0.0079*), Dunn´s post test. C- PARP expression at S at analyzed groups (p=0.0042 Kruskal-Wallis test). Group C versus AI (p=0,0079**); group A versus AI (p=0,0079*), Dunn´s post test. D- AIF protein expression at CDL at analyzed groups (p=0.0003 Kruskal-Wallis test). E- AIF protein expression at CL at analyzed groups (p=0.0187 Kruskal-Wallis test). There was not statistical difference among the groups. F- AIF protein expression at S at analyzed groups (p=0.0187 Kruskal-Wallis test). There was not statistical difference among the groups.

It was noted positive stain in all the groups, but group AI presented the lowest one (Fig. 2).

I was seen positive nuclear stain for AIF protein in all the groups analyzed at CDL and CL, exclusively at S for the group AI. The comparison among the groups identified an increase of AIF protein expression in animals from group AI when compared to the others (Figs. 1 D, E, F). It was seen positive stains only for the group AI (Fig. 2).

MiRNA-9 serum genic expression analysis. The serum expression of miRNA-9 and was low for all the groups, being the highest one for animals from group C, but without statistical significance (p=0,3309, Kruskal-Wallis test).

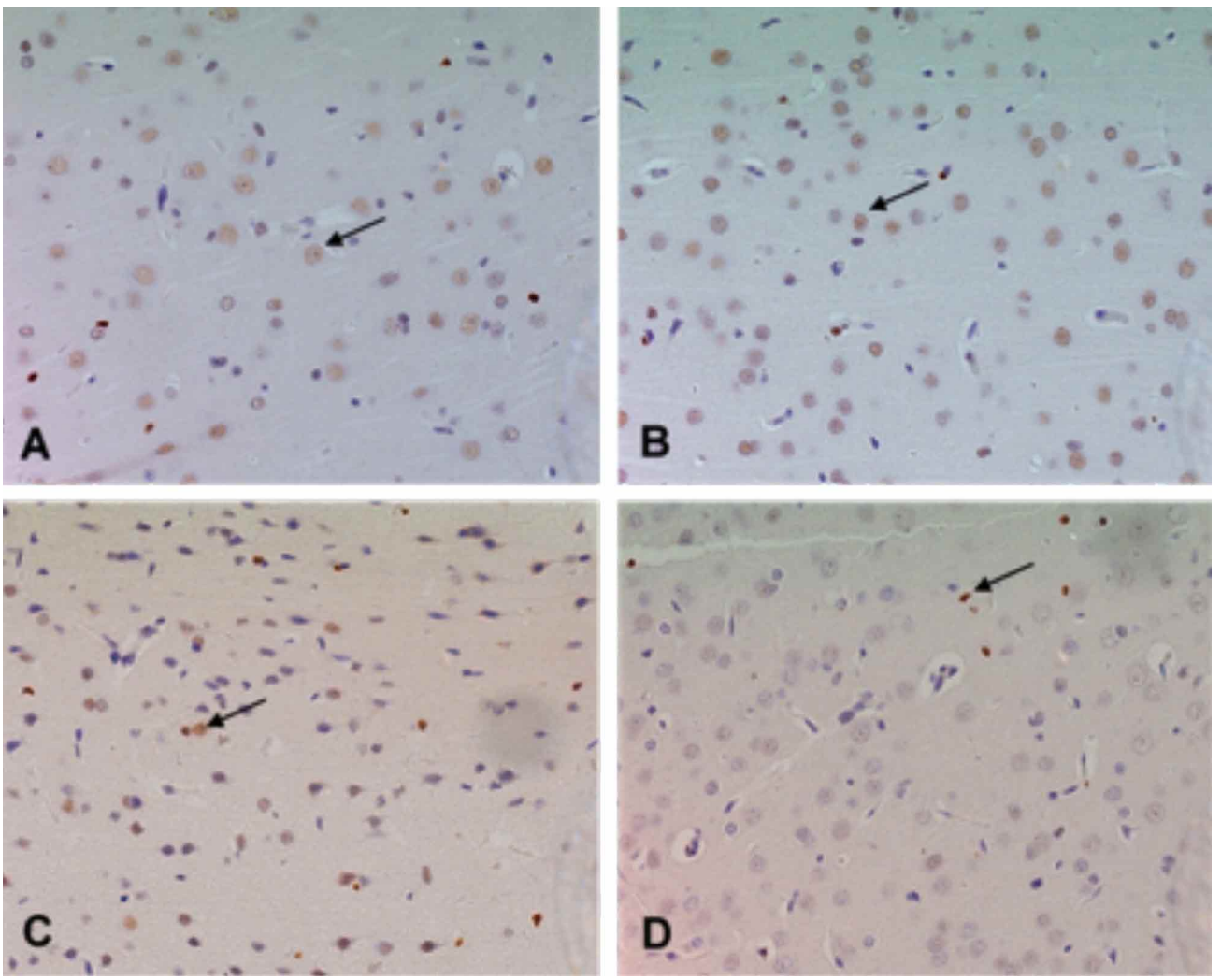

Fig. 2. Photomicrograph of dorsolateral cortex (CDL) of PARP-1 immunohistochemistry. A: group C; B: group A; C: group I; D: group AI. Positively stained cells for PARP-1 are identified by the arrows. The highest positive stain was found at group AI. 400x.

DISCUSSION

In the cerebral ischemia the inhibition of signaling pathways involved in oxidative stress, inflammatory responses, and subsequent damage of neuronal apoptosis and death (Abas *et al.*).

Evidence suggests that in order to achieve post-ischemic neuroprotection *in vivo* it is imperative to save not only neurons, but also other types of cells present in brain tissue. For this, it is important to identify suitable targets possibly expressed in most cell types.

In recent years, some PARP-1 inhibitors, given after cerebral ischemia, have emerged as interesting tools to reduce post-ischemic brain damage. Some laboratory studies

have reported that PARP activation may be detrimental to most brain cells, and PARP inhibitors may reduce tissue damage, improving survival of neurons and glial cells among others (Moroni & Chiarugi, 2009).

In the present study, the expression of the PARP-1 enzyme was increased after the ischemia process, although protein expression was not always altered (O'Keefe *et al*.).

Slight DNA damage occurs, PARP-1 activation results in poly (ADP-ribosylation) alone, histones, and other factors involved in DNA repair and control transcriptional. These effects lead to the repair of DNA lesions and cell survival. In contrast, excessive DNA damage leads to the hyperstimulation of PARP-1 which produces large amounts of PAR polymers, leading to the translocation of AIF and to the process of apoptosis independent of caspase or necrosis (Heeres & Hergenrother).

Analyzing our results we can suggest that low expression of AIF in group I indicates that the ischemia model possibly generated slight DNA damage which stimulated the activation of PARP-1 only at levels sufficient to promote DNA repair and not the activation of the process of apoptosis by the translocation of AIF. As demonstrated in our results, the AI group showed a significant reduction in the protein expression of PARP-1 when compared to the other groups. On the other hand, there was no protein expression of AIF in any of the other groups, being only expressed in the AI group. No data were found in literature that indicated reduced PARP-1 expression in I / R models associated with chronic alcohol use.

In experimental models, the use of ethanol was sufficient to cause substantial brain neurodegeneration. In this way, PARP-1 is linked to the cell death programs and is triggered by oxidative stress caused by exposure to ethanol. Our data still differed with other authors who had increased levels of PARP-1 in adult rats submitted to the use of chronic ethanol but who did not undergo the I / R model (Natarajan *et al*., 2015).

In this principle, miRNA-9 is one of the responsible for regulating the serum response factor (FRS) in vitro. FRS is activated in response to ischemia and stimulates precursor cells (CPOs) to differentiate into mature oligodendrocytes (OLs). These cells are responsible for the formation of the myelin sheath in an attempt to preserve neurological function, indicating that miRNA-9 may be involved in the regulation of FRS through an ischemic process (Sallmann *et al*.).

In our study, miRNA-9 expression did not present a statistically significant difference between the groups, however it can be observed that groups A, I and AI presented lower expression when compared to group C.

Our data corroborate with other studies, which demonstrated that high doses (320mg / dl) of ethanol suppress the expression of specific miRNAs during cortical neurogenesis (miRNA-21, miRNA-335, miRNA-9 and miRNA 153) (Hassa *et al*.).

No data were found in the literature related to miRNA-9 where there was association of the I / R mechanism associated with chronic alcohol use, however, our results suggest that the association of these two factors did not provide significant alteration in miRNA-9 expression.

Alcoholism is a multigenic disease in which large gene networks are affected. Considering the general effect of miRNA-9 on the expression of several genes relevant to ethanol actions on the CNS, it is tempting to speculate that miRNA-9 plays a substantial role in mediating the effects of ethanol on the development of alcoholism. (Griffiths-Jones *et al*., 2006).

CONCLUSION

This study concluded that the protein expression of PARP-1 was decreased in all brain areas analyzed in the ischemic group associated with alcoholism. On the other hand, the protein expression of AIF increased in these same areas.

No correlation was observed between the expression of miRNA-9 with the protein expression of PARP-1 and AIF, since miR-9 showed low levels of expression in all groups when compared to the control group.

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RESUMEN: El consumo crónico de alcohol provoca un empeoramiento de los eventos que siguen a la isquemia cerebral. Estos eventos están regulados a través de la expresión de varios genes y microRNA. El objetivo de este trabajo fue analizar y describir el perfil de expresión de las proteínas PARP y AIF y microRNA-9 en ratas sometidas a isquemia cerebral focal, asociadas o no, con el modelo de alcoholismo crónico. Veinte ratas Wistar adultas se dividieron en: grupo control, isquémico alcohólico, e isquémico / alcoholizado para análisis inmunohistoquímico y ex-

presión de genes microRNA-9. Resultados: Hubo una reducción en la expresión de proteínas de PARP-1 y un marcado positivo para AIF en el grupo isquémico / alcoholizado. No se observó una expresión significativa en el microRNA-9. La asociación de la isquemia con el consumo crónico de alcohol promovió una tendencia a la baja expresión de microRNA-9, baja expresión de PARP-1 y alta expresión de AIF, lo que indica una interferencia en el efecto protector de microRNA-9 en los otros grupos .

PALABRAS CLAVE: PARP-1; AIF; microRNA-9; Isquemia cerebral; Alcoholismo.

REFERENCES

Abas, F.; Alkan, T.; Goren, B.; Taskapilioglu, O.; Sarandol, E. & Tolunay, S. Neuroprotective effects of postconditioning on lipid peroxidation and apoptosis after focal cerebral ischemia/reperfusion injury in rats. *Turk. Neurosurg., 20(1)*:1-8, 2010.

Carlotti Junior, C. G.; Colli, B. O. & Kazuo, J. Y. Avaliação da isquemia cerebral pela respiração mitocondrial. *Arq. Neuro-Psiquiatr., 59(2B)*:365-71, 2001.

David, K. K.; Andrabi, S. A.; Dawson, T. M. & Dawson, V. L. Parthanatos, a messenger of death. *Front. Biosci. (Landmark Ed.), 14*:1116-28, 2009.

Donnan, G. A.; Fisher, M.; Macleod, M. & Davis, S. M. Stroke. *Lancet, 371(9624)*:1612-23, 2008.

Gibson, G. E.; Ratan, R. R. & Beal, M. F. Mitochondria and oxidative stress in neurodegenerative disorders. Preface. *Ann. N. Y. Acad. Sci., 1147(1)*:11-2, 2008.

Ginsberg, M. D. Neuroprotection for ischemic stroke: past, present and future. *Neuropharmacology, 55(3)*:363-89, 2008.

Goldstein, L. B. & Rothwell, P. M. Advances in prevention and health services delivery 2010–2011. *Stroke, 43*:298-9, 2012.

Griffiths-Jones, S.; Grocock, R. J.; van Dongen, S.; Bateman, A. & Enright, A. J. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res., 34(Database issue)*:D140-4, 2006.

Hassa, P. O.; Haenni, S. S.; Elser, M. & Hottiger, M. O. Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going? *Microbiol. Mol. Biol. Rev., 70(3)*:789-829, 2006.

Heeres, J. T. & Hergenrother, P. J. Poly(ADP-ribose) makes a date with death. *Curr. Opin. Chem. Biol., 11(6)*:644-53, 2007.

Lee, J. H.; Kim, K. Y.; Lee, Y. K.; Park, S. Y.; Kim, C. D.; Lee, W. S.; Rhim, B. Y. & Hong, K. W. Cilostazol prevents focal cerebral ischemic injury by enhancing casein kinase 2 phosphorylation and suppression of phosphatase and tensin homolog deleted from chromosome 10 phosphorylation in rats. *J. Pharmacol. Exp. Ther., 308(3)*:896-903, 2004.

Lin, W.; Amé, J. C.; Aboul-Ela, N.; Jacobson, E. L. & Jacobson, M. K. Isolation and characterization of the cDNA encoding bovine poly(ADP-ribose) glycohydrolase. *J. Biol. Chem., 272(18)*:11895-901, 1997.

Mandel, M.; Fonoff, E. T.; Bor-Seng-Shu, E.; Teixeira, M. J. & Chadi, G. Neurogenic neuroprotection: clinical perspectives. *Funct. Neurol., 27(4)*:207-16, 2012.

Mehta, S. L.; Manhas, N. & Raghubir, R. Molecular targets in cerebral ischemia for developing novel therapeutics. *Brain Res. Rev., 54(1)*:34-66, 2007.

Moroni, F. & Chiarugi, A. Post-ischemic brain damage: targeting PARP-1 within the ischemic neurovascular units as a realistic avenue to stroke treatment. *FEBS J., 276(1)*:36-45, 2009.

Natarajan, S. K.; Pachunka, J. M. & Mott, J. L. Role of microRNAs in alcohol-induced multi-organ injury. *Biomolecules, 5(4)*:3309-38, 2015.

O'Keefe, J. H.; Bhatti, S. K.; Bajwa, A.; DiNicolantonio, J. J. & Lavie, C. J. Alcohol and cardiovascular health: the dose makes the poison…or the remedy. *Mayo Clin. Proc., 89(3)*:382-93, 2014.

Sallmann, F. R.; Vodenicharov, M. D.; Wang, Z. Q. & Poirier, G. G. Characterization of sPARP-1. An alternative product of PARP-1 gene with poly(ADP-ribose) polymerase activity independent of DNA strand breaks. *J. Biol. Chem., 275(20)*:15504-11, 2000.

Sobrado, M.; López, M. G.; Carceller, F.; García, A. G. & Roda, J. M. Combined nimodipine and citicoline reduce infarct size, attenuate apoptosis and increase bcl-2 expression after focal cerebral ischemia. *Neuroscience., 118(1)*:107-13, 2003.

Sriram, C. S.; Jangra, A.; Gurjar, S. S.; Hussain, M. I.; Borah, P.; Lahkar, M.; Mohan, P. & Bezbaruah, B. K. Poly (ADP-ribose) polymerase-1 inhibitor, 3-aminobenzamide pretreatment ameliorates lipopolysaccharide-induced neurobehavioral and neurochemical anomalies in mice. *Pharmacol. Biochem. Behav., 133*:83-91, 2015.

Wang, C. P.; Shi, Y. W.; Tang, M.; Zhang, X. C.; Gu, Y.; Liang, X. M.; Wang, Z. W. & Ding, F. Isoquercetin ameliorates cerebral impairment in focal ischemia through anti-oxidative, anti-inflammatory, and anti-apoptotic effects in primary culture of rat hippocampal neurons and hippocampal CA1 region of rats. *Mol. Neurobiol., 54(3)*:2126-42, 2017.

Yu, S. W.; Andrabi, S. A.; Wang, H.; Kim, N. S.; Poirier, G. G.; Dawson, T. M. & Dawson, V. L. Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death. *Proc. Natl. Acad. Sci. U. S. A., 103(48)*:18314-9, 2006.

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