

Characterizing and Profiling microRNAs in Dogs Undergoing Induced Ischemic Brain Stroke After Middle Cerebral Artery Occlusion Under Fluoroscopic Guidance

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Purpose: Ischemic stroke of the brain is still considered one of the most common causes of disability and death in developed and developing countries in human beings despite advances in medicine and technology. This study was conducted to characterize and profile tens of induced biomarkers (microRNAs) after experimentally inducing regional ischemic stroke of the brain by occluding the middle cerebral artery under fluoroscopic guidance using an autologous blood clot.

Patient and Methods: A total of six healthy dogs were recruited for this study. The microRNAs were profiled in the blood and urine before and after occluding the middle cerebral artery using genetic techniques.

Results: The very highly expressed genes were comprised within cluster A, followed by cluster D in both 24 and 48-hour brain samples. Clusters B and C revealed down-regulated genes, while miRNAs remained up-regulated in the 24-hour samples merely in cluster F. Upregulated genes at 48 hours of reperfusion were included in cluster E. On the other hand, changes were observed after a day on the cluster G genes. Exclusive upregulation was notified after 2 days due to the changes in miR-138. The normalized gene expression in the test sample is witnessed through Fold-Change, which divides the control sample's normalized gene expression. Moreover, fold-change has emerged as a significant approach for representing fold-regulation.

Conclusion: The microRNAs expression in blood and urine may have a potential role in the diagnosis, prognosis, and assessment of therapy associated with cerebral artery occlusion under fluoroscopic guidance.

Keywords: dogs, fluoroscopic induced ischemic brain, microRNAs, middle cerebral artery stroke

Introduction

Ischemic stroke of the brain is considered one of the challenging causes of short- or long-term neurological disability and one of the most frequent causes of mortality in human medicine worldwide. The most commonly implicated ischemic stroke in human medicine includes embolic or thrombotic obstruction of major cerebral arteries, such as the middle cerebral artery.^{1,2} It has been estimated in 2008 that ischemic stroke did account for an estimated cost of over 65 billion USD. The confirmed incidence of stroke has been estimated to be approximately about 10% and this is on the rise due to

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many factors. In veterinary medicine, the incidence and prevalence rates are undocumented, but this disease identity is becoming more recognized with advanced development and widespread availability of more advanced diagnostic imaging techniques, such as contrast magnetic resonance imaging.

The exact etiology and pathogenesis of brain stroke are still not fully understood. There are many risk factors associated with stroke, including age, hypertension, diabetes mellitus, and obesity. The clinical manifestation and variability of clinical signs associated with ischemic stroke of the cerebrum depends on many factors but largely on the location of ischemia, the severity of the ischemia, complexity of the ischemia, age, presence of cancer, and potentially concurrent diseases.^{1,3–6}

This study has been proposed to induce ischemic stroke of the cerebrum by selectively and mechanically occluding the middle cerebral artery (MCA). Selective occlusion of the MCA has been suggested to induce focal-type ischemia.⁷ This model mimics the pathophysiology of ischemic stroke in humans. Occlusion of the MCA was performed using autologous blood clots under aseptic technique through the right femoral arterial access under fluoroscopy guidance. Fluoroscopy is a real-time dynamic X-ray machine that will help in navigating the location of the MCA, thus allowing to occlude the described artery.

A group of regulatory aspects is widely represented through microRNAs for facilitating cells to furnish multifaceted gene expression cascades in biological procedures.⁸ MicroRNAs have been found in many tissues, including blood and body fluids, such as cerebral spinal fluids, in stable forms that are protected from endogenous RNase activity. Characterizing and profiling microRNAs is considered an innovative technique to identify biomarkers associated with many diseases. For instance, microRNAs (miR 206, miR 574-5p) have been proven to be a powerful tool in the diagnosis and treatment of many cardiac diseases including coronary artery diseases.⁸ Researchers have pinpointed other significant microRNAs (miR-125b-2*-27a*miR-210, -422a, -488, and -627) that were considered to be of significant values in patients with cardiovascular and ischemic conditions.

Here, the main aim is to characterize blood microRNAs before and after experimental induction of cerebral ischemia. The secondary objectives of this study include profiling urine microRNAs in dogs undergoing experimental induction of cerebral stroke by occluding the middle cerebral artery under fluoroscopic guidance.

To the authors' knowledge, this is considered the first publishable research effort to experimentally induce acute cerebral ischemia in dogs by characterizing and profiling potential microRNAs biomarkers in blood and urine and concurrently measuring cardiac troponin in this group of dogs. Knowledge of the biomarkers that can potentially be of clinical significance in patients with acute cerebral ischemia can be a novel approach to accurately utilize in diagnosis and evaluate the effectiveness of treatment, thereby lowering mortality and morbidity in such group of patients.

Patient and Methods

In veterinary care were followed, and that the client/owner provided informed consent for their pet/animal to be used in this study. A total of 6 healthy middle-aged dogs (1–5 years) were recruited for this study. The dogs were obtained from local breeders. Dogs underwent complete physical examination, complete blood count (platelet counts, white and red blood cell counts), serum biochemistry panel (urea, creatinine, liver enzymes [ALT, ALP, GGT], glucose, albumin, globulin) to evaluate the function of internal organs to ensure general health status. Only healthy dogs were included in this study. Dogs with historical or clinical signs suggestive of internal organ dysfunction were excluded from the study. Obese and aggressive dogs were not considered for this project. Selected animals were vaccinated against canine distemper, adenovirus, parvovirus, *Leptospira* species and rabies [Vanguard[®] and Rabies[®] and *Leptospira*] at the time of admission to the hosting facility. In addition, a deworming regimen using Ascaten[®] (1 tab/5kg) was started and repeated in 2 weeks. Dogs were housed at the hosting facility for 14 days before the start of the experimental design. Each dog was given Collar ID and was housed separately in cages and provided with daily dry food, and ad-lib source of water. The dogs were fed commercially available food (BensonTM). The main purpose of this pre-experimental housing was to give ample time for dogs to adjust and acclimatize to the new environment and to observe any developing abnormally physical or behavioral manifestation that could not be identified at the time of selection.

Selected dogs were anesthetized using intravenous Propofol (4–6 mg/kg). Selected dogs underwent arterial catheterization of the right femoral artery under strict aseptic technique and under fluoroscopic guidance to navigate around to selectively occlude the middle cerebral artery. Measuring microRNAs and data analysis: Assay Kits were used to purify microRNA and total RNA from the collected

urine and serum samples according to the manufacturer's instructions and were stored aliquoted in RNase/DNase-free water at -80°C . The total RNA and microRNA quality and quantity were checked using both bio-analyzer 2100 (Agilent) and Nano-DropTM 1000 Spectrophotometer (Thermo Scientific). The TaqMan Human MicroRNA Assay Set (Applied Biosystems) consisting of Sanger database unique assays to quantify Stroke microRNAs and 10 controls (Z30 and nine different SNORs/RNUs) were used to screen a set of 20 strokes and 20 normal specimens for differentially expressed microRNAs.

The reliance of TaqMan MicroRNA Reverse Transcription assay is on a particular hairpin structure throughout cDNA synthesis and mature microRNA particular detection probes. These aspects preclude the identification of precursor microRNAs. To be precise, pooled analysis for different RT primers was done based on the suggestions given for 30 min in a speed vacuum centrifuge and resuspended for an ultimate concentration of 50 nM each. The final volume was reverse-transcribed through 25 to 50 ng of total RNA sample aliquots using the TaqMan MicroRNA Reverse Transcription Kit.

Blood was collected from the jugular or cephalic vein to profile the microRNAs. Descriptive statistics were performed for each of the listed variables. Data were analyzed with one-way ANOVA, using GraphPad Prism 5.0 software (GraphPad Prism, San Diego, CA) and GenEx software (www.multid.se) or REST V3. The significant difference was set at a P value less than 0.05. Relative quantification of microRNA expression was calculated using the comparative Ct method (Applied Biosystems User Bulletin N2 (P/N 4303859)). For proper comparison of microRNA, expression data were normalized with appropriate reference genes using geNorm algorithms. The microRNAs were serially evaluated: one analysis before the arterial collusion and 3 analyses post occlusions (1-hour post, 4 hours post, 1-day s post).

The project has no special safety considerations. Dogs determined to fulfill the criteria of the study were housed at the Veterinary Health Center at Jordan University of Science and Technology. Individual animal subjects were housed in separate and clean cages with free access to water and a standard feeding program. Dogs were fully vaccinated to eliminate any potential zoonotic hazards. A portable fluoroscopy machine was housed in the lead radiology room for the study duration at the veterinary teaching hospital. Urine samples were collected regarding microRNA.

Results

A total of 6 middle-aged dogs were screened based on brain and blood samples subjected to microRNA. Very few microRNA transcripts were identified in the urine and blood, and the majority of the transcripts were identified in the brains of dogs for 24 and 48 hours, respectively. Hierarchical clustering of the microRNAs identified in the brain revealed 7 different clusters. The very highly expressed genes were comprised within cluster A, followed by cluster D in both 24 and 48-hour brain samples. Clusters B and C revealed the down-regulated genes while miRNAs remained up-regulated in the 24-hour samples merely in cluster F. Upregulated genes at 48 hours of reperfusion were included in cluster E. On the other hand, changes were observed after a day on the cluster G genes. Exclusive upregulation was notified after 2 days due to the changes in miR-138.

Tables 1 and 2 show the list of genes and plate-position selected for both groups in serum and urine profile, respectively. The selection was made after elevation in serum to increase expression from 2 h to 24 h.

Fold Change and Fold Regulation (Serum Profile)

The normalized gene expression in the test sample is witnessed through Fold-Change, which divides the control sample's normalized gene expression. Moreover, fold-change has emerged as a significant approach for representing fold-regulation, as shown in Tables 3 and 4. The values are shown to a greater extent in the fold-change, indicating a positive or an up-regulation, whereas the fold-regulation is similar to the fold change. As shown in Figures 1 and 2, a negative or down-regulation is represented through fold-change values less than 1, where both urine and serum profiles showed a negative inverse of the fold-change.

Discussion

The role of miRNAs in many disease disorders has been studied in recent years. Many miRNAs have important roles in the cell cycle, hematopoiesis, and cellular metabolic control.⁹ Several *in vitro*^{10–12} and *in vivo* (human)^{13,14} investigations have demonstrated that miRNAs are temporally modulated not only in the brain but also in the peripheral blood during the course of and notably during the reperfusion phase of a cerebral ischemic event. Differentially expressed miRNAs have been linked to inflammation, endothelial/vascular function, erythropoiesis, angiogenesis, brain function,

Table 1 List of Genes and Plate-Position for Both Groups in Our Data (Serum Groups)

Plate-Position	Mature ID	Fold Change	95% CI	Fold Change	95% CI	Rank
1-A06	cfa-miR-1	0.611	(0.00001, 1.63)	0.2068	(0.00001, 0.48)	105
2-C04	cfa-miR-628	1.6681	(0.00001, 4.18)	5.7932	(0.00001, 13.64)	127
2-D02	cfa-miR-384	0.1977	(0.00001, 0.63)	0.3195	(0.00001, 1.31)	137
2-F07	cfa-miR-490	0.0876	(0.00001, 0.27)	0.0716	(0.00001, 0.35)	167
2-G05	cfa-miR-424	0.3155	(0.08, 0.55)	0.5982	(0.27, 0.93)	176
2-G06	cfa-miR-300	0.2365	(0.00001, 0.85)	0.1448	(0.00001, 0.51)	177
3-D01	cfa-miR-450a	1.4977	(0.23, 2.76)	3.6303	(0.84, 6.42)	232
3-E10	cfa-miR-371	0.9568	(0.31, 1.60)	1.9687	(0.90, 3.04)	253
3-F06	cfa-miR-132	0.0657	(0.00001, 0.19)	0.1319	(0.00001, 0.32)	261
4-B03	cfa-miR-758	1.2986	(0.00001, 3.57)	14.7391	(0.00001, 39.38)	306

Table 2 List of Genes and Plate-Position for Both Groups in Our Data (Urine Groups)

Plate-Position	Mature ID	Fold Change	95% CI	Fold Change	95% CI	Rank
1-C02	cfa-miR-148a	14.2322	(0.00001, 36.34)	4.9278	(0.00001, 14.91)	29
1-F04	cfa-miR-27b	6.0845	(0.57, 11.60)	1.3753	(0.08, 2.68)	67
1-G07	cfa-miR-499	8.0138	(0.00001, 16.74)	3.9822	(0.00001, 18.36)	82
2-A08	cfa-miR-874	0.1532	(0.00001, 0.64)	0.1031	(0.00001, 0.33)	107
2-F10	cfa-miR-138b	5.5149	(0.00001, 15.16)	0.0896	(0.00001, 0.40)	169
3-A10	cfa-miR-363	9.0864	(0.00001, 21.14)	0.1526	(0.00001, 0.70)	205
3-B03	cfa-miR-487b	10.6105	(0.00001, 22.24)	0.1931	(0.00001, 0.95)	210
4-B05	cfa-miR-491	0.3482	(0.00001, 0.77)	0.1666	(0.06, 0.27)	308

and hypoxia in people who have had a stroke.^{13–15} Therefore, we conducted this study to experimentally induce acute cerebral ischemia in dogs to characterize and profiling potential microRNAs biomarkers in blood and urine samples.

When we look at the microRNA of urine, the sample elevates after 2 hours and then decreases expression at 24 hours. On the contrary, the serum elevated to increase expression from 2 hours to 24 hours. The urine will be a tremendous indication in the case model and the performance of the molecular pathology circulation differs in serum and urine. At this point, the microRNA circulated in serum, an effect of molecular pathology in urine after 2 hours, and then the return to normal. On the other hand, the microRNA serum will elevate and reflect the destruction (Dog Model) appearing to increase the expression of miRNA.

A multifaceted cascade of biochemical and physiologic events was triggered by cerebral ischemia. It was assumed that these events were mediated in part by variations in molecular procedures including translation and transcription. In this regard, Lu et al¹⁶ detected 12 functional categories, contributing to the temporal modifications in gene expression after transient microRNAs in dogs. To date, there were no reports available on the microRNA microarray profiling of the ischemic brain. Nonetheless, the roles of particular microRNAs have been explained in several reports regarding neurogenesis, neural cell specification, neurodevelopmental function, and neuronal differentiation.¹⁷ The expression of 114 miRNAs in ischemic brain samples was identified. Out of these, 82 transcripts and 106 transcripts were detected in the 48-hour and 24-hour reperfusion brain samples, respectively.¹⁸

Table 3 Fold Regulation for Serum Groups

Plate-Position	Mature ID	p-value (Comparing to Control Group)	Fold Regulation
1-A06	cfa-miR-1	0.041577	-4.8345
2-C04	cfa-miR-628	0.030319	5.7932
2-D02	cfa-miR-384	0.047123	-5.0577
2-F07	cfa-miR-490	0.044611	-11.4194
2-G05	cfa-miR-424	0.003788	-3.1697
2-G06	cfa-miR-300	0.028841	-4.2288
3-D01	cfa-miR-450a	0.033694	3.6303
3-E10	cfa-miR-371	0.044262	1.9687
3-F06	cfa-miR-132	0.02277	-15.2135
4-B03	cfa-miR-758	0.047277	14.7391

Table 4 Fold Regulation for Urine Groups

Plate-Position	Mature ID	p-value (Comparing to Control Group)	Fold Regulation
1-C02	cfa-miR-148a	0.011912	14.2322
1-F04	cfa-miR-27b	0.014591	6.0845
1-G07	cfa-miR-499	0.014423	8.0138
2-A08	cfa-miR-874	0.046449	-9.7029
2-F10	cfa-miR-138b	0.038931	5.5149
3-A10	cfa-miR-363	0.008181	9.0864
3-B03	cfa-miR-487b	0.001023	10.6105
4-B05	cfa-miR-491	0.046796	-6.0033

The data have revealed that microRNAs were actively regulated and that their expression pattern was modified with reperfusion time, indicating temporal expression throughout the ischemic injury.¹⁹ The majority of the reports have indicated that prolonged reperfusion time was increased with neuronal damage, and this damage can be more extreme as compared to the ischemic injury itself.²⁰ Different upregulations were further observed at 24 and 48 hours of reperfusion for miR-138 and -145, respectively.²¹ The developmental regulation of miR-138 has been confined to different neuronal populations in the midbrain, cerebrum, and cerebellum of dogs, while miR-145 has been recognized as a particular miRNA that was down-regulated in cancers.²²

A total of 32 unique transcripts were represented in the 24-hour brain samples while only 8 unique detectable miRNAs were found in the 48-hour brain samples. A high expression was identified for miR-290 at 24 hours and elevated by 5 times at the 48-hour reperfusion time. The highest expression was revealed by the miR-292-5p after ischemic injury in the brain at 24-hour reperfusion, and elevated even more, by 1.5 times, at 48-hour reperfusion. The majority of the genes, as per DNA microarray data, revealed that miR-290 was downregulated. In brain samples at 24-hour reperfusion, miR-214 was highly expressed but was reduced in the 48-hour reperfused brain.

Because ischemic stroke in people is a diverse illness with complicated pathophysiology, it is impossible to mimic all characteristics of human stroke in a single animal model.

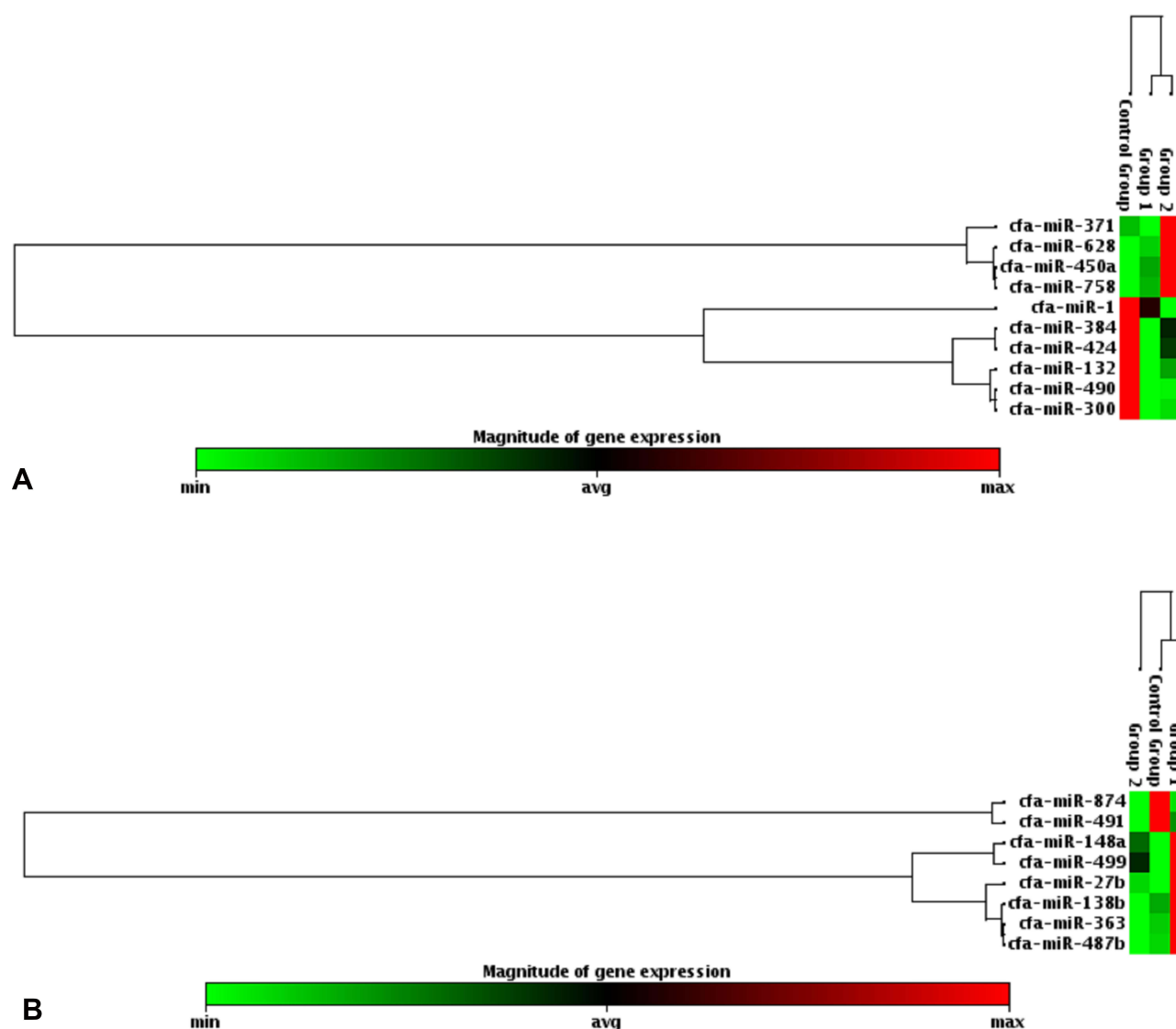


Figure 1 Fold change regulation: (A) Serum profile; (B) Urine profile.

One of the models that most accurately simulate human ischemic stroke is the transient or permanent middle cerebral artery blockage (MCAo). Furthermore, the infarcts in this model are consistent and repeatable. Therefore, the MCAo model is considered for this study. The MCAo model offers several benefits including its close resemblance to human ischemic stroke, which is frequently caused by MCAo and has a penumbra similar to that of human stroke. The MCAo model is also notable for its enormous infarct size and good repeatability. Reperfusion and, as a result, the length of ischemia may be carefully controlled. Furthermore, the process is simple to carry out and does not take long. The MCAo model is thought to be appropriate for replicating ischemic stroke and

associated neuronal cell death, cerebral inflammation, and BBB damage, as well as providing acceptable behavior test outcomes.^{23,24}

Given the described study design, the dogs are expected to finish the experimental period. The findings will, for the first time, estimate and profile the induced biomarkers potentially associated with experimentally induced cerebral ischemia achieved by non-invasively occluding the middle cerebral artery utilizing advanced interventional neuroradiology techniques. Findings to be obtained from this study may potentially be translated into human medicine that will help in the diagnosis treatment and evaluation of treatment intervention.

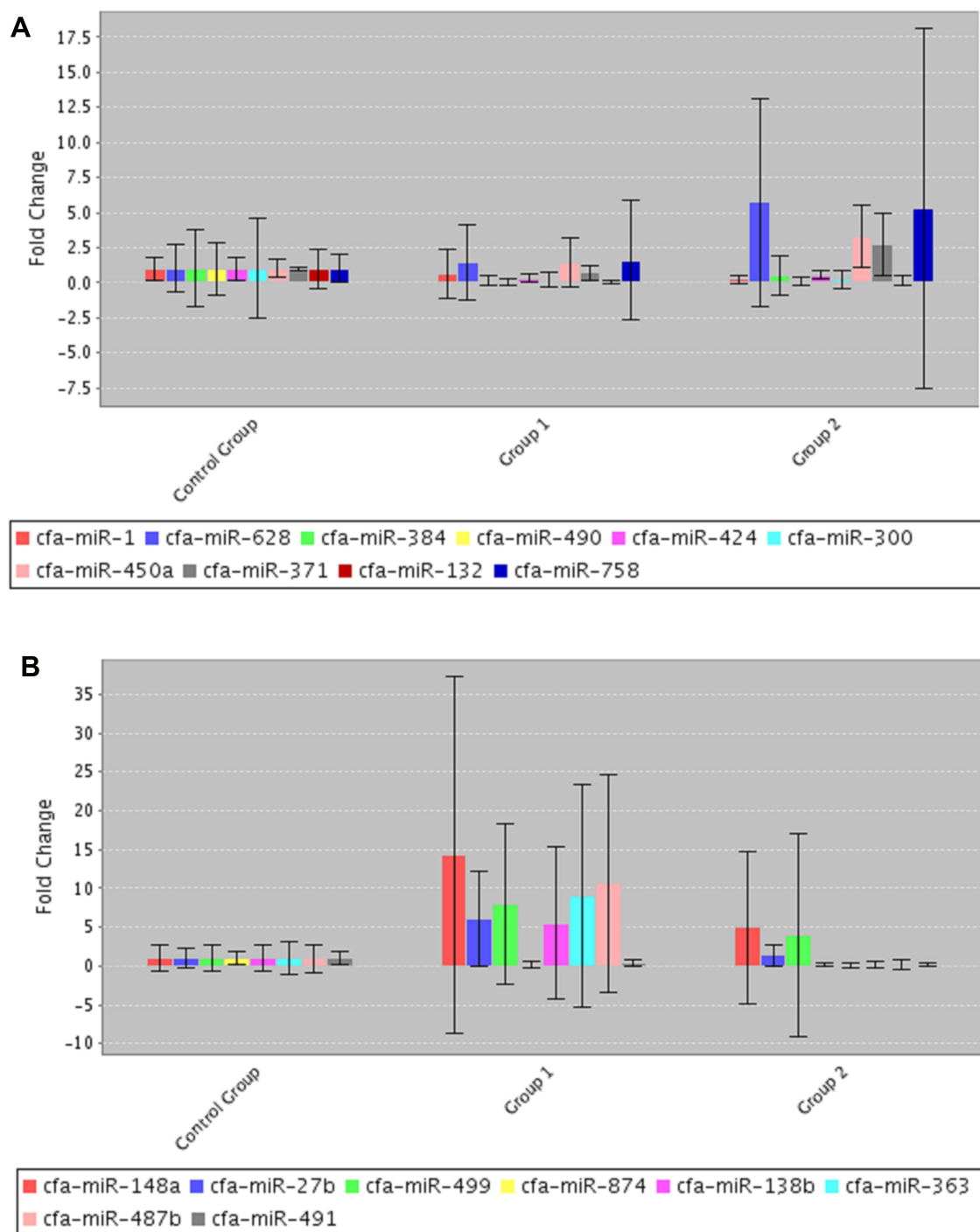


Figure 2 Magnitude of gene expression: (A) Serum profile; (B) Urine profile.

Conclusion

This study has presented the profiling of miRNA throughout ischemia and reperfusion in a dog model and has detected different regulation patterns for seven clusters of miRNA. This study has further associated the outcomes with appropriate

DNA microarray data and has detected some of the miRNAs that control the expression of 4 genes identified to be essential for the development of cerebral ischemia. The findings might be beneficial to seek additional studies on the probable use of microRNAs as biomarkers for stroke.

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Disclosure

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