



Article

Molecular Investigation of DKK3 in Cerebral Ischemic/Reperfusion Injury

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Abstract: Dickkopf-3 (Dkk3) is an atypical member of the Dkk family of Wnt inhibitors, which has been implicated in the pathophysiology of neurodegenerative disorders. Its role in the mechanisms of cellular degeneration and protection is still unknown. The aim of our work is to investigate the endogenous activation of the DKK3 pathway in a model of transient occlusion of the middle cerebral artery in rats. In particular, the animals were subjected to 1 h of ischemia followed by different reperfusion times (1 h, 6 h, 12 h and 24 h) to evaluate the downstream pathway and the time course of its activation. Western blot analysis showed increased Dkk3 expression in animals with the highest time of reperfusion. The increased levels of Dkk3 were accompanied by reduced Wnt3a, Frz1 and PIWI1a expression in the cytosol while FOXM1 and β -catenin decreased in the nucleus. These molecular changes led to an increase in the apoptotic pathway, as showed by the increased expression of Caspase 3 and Bax and the reduced levels of Bcl-2, and to a decrease in neurogenesis, as shown by the decreased expression of Tbr2, Ngn2 and Pax6. In the second part of the study, we decided to employ curcumin, an activator of the Wnt/ β -catenin signaling, to investigate its effect on Dkk3. In particular, curcumin was administered 1 and 6 h after ischemia, and animals were sacrificed 24 h later when the expression of Dkk3 was higher. Our data displayed that curcumin administration decreased Dkk3 expression, and increased Wnt3a, Frz1 and PIWI1a levels. Well in line with these data, curcumin administration increased nuclear β -catenin and FOXM1 expression. The down-regulation of Dkk3 by curcumin led to reduced apoptosis and increased neurogenesis. Summarizing, our results showed that Dkk3 acts as an inhibitor of Wnt/ β -catenin signaling during cerebral ischemia. Additionally, its inhibition and the contextual activation of the Wnt/ β -catenin pathway are protective against ischemic stroke.

Keywords: stroke; ischemia/reperfusion; DKK3



Citation: Caffo, M.; Fusco, R.; Siracusa, R.; Caruso, G.; Barresi, V.; Di Paola, R.; Cuzzocrea, S.; Germanò, A.F.; Cardali, S.M. Molecular Investigation of DKK3 in Cerebral Ischemic/Reperfusion Injury. *Biomedicines* **2023**, *11*, 815. <https://doi.org/10.3390/biomedicines11030815>

Academic Editor: Juan Sahuquillo

Received: 17 February 2023

Revised: 1 March 2023

Accepted: 4 March 2023

Published: 7 March 2023



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1. Introduction

Stroke is a rapid alteration of cerebral blood supply that induces disturbance of cerebral function. It should be divided into two types: ischemic and hemorrhagic with, respectively, 15 and 85% incidence [1,2]. Ischemic stroke is the third cause of mortality worldwide and the first cause of disability. Nevertheless, ischemic stroke still needs a treatment that has beneficial outcomes. In the acute phase of the event, there is glucose and oxygen depletion in the brain, a reduction in blood flow that alters ionic homeostasis, and an increase in reactive oxygen species (ROS) generation and intracellular calcium stress responses [3]. Additionally, inflammatory and apoptotic pathways lead to neuronal cell death and loss of biological functions [4]. Thus, one of the main goals of stroke treatment is the protection of

neurons [5,6]. Of particular interest, researchers have underlined the activation of intrinsic and extrinsic pathways of cell death [7]. In the activation of the intrinsic pathway, the permeabilization and depolarization of the mitochondrial membrane and the release of proapoptotic mediators from the mitochondrial space are involved [8]. In particular, due to mitochondrial injury, cytochrome c is released leading to the proteolytic activation of caspases [9]. Among them, it has been described that the activation of caspase 3 is strongly increased in experimental models of ischemic stroke [7]. Wnt/ β -catenin signaling has shown protective effects in the brain after traumatic brain injury [10]. It displayed neuroprotective effects preserving neurogenesis and having anti-apoptotic and anti-inflammatory properties [11]. In particular, in absence of Wnt ligands, β -catenin expression is reduced due to proteasome degradation, while in the presence of Wnt ligands, β -catenin levels increase. In this case, β -catenin translocates into the nucleus and initiates the transcription of Wnt target genes, including FOXM1, which blocks Bax and caspase 3 activation and increases Bcl-2 expression [12,13]. In addition, β -catenin can combine with transcription factors of the T-cell factor/lymphoid enhancer factor (Tcf/Lef) family and manage the expression of proteins such as Pax 6, neurogenin 2 and Tbr 2 [14–16]. The Wnt pathway is regulated by the Dickkopf (Dkk) family, which includes four members (Dkk1 to 4) of which Dkk3 differs from the others [17]. Different from the proteins of the same family, the modulatory effects of Dkk3 on the Wnt pathway are context-dependent [18]. Several studies have displayed that Dkk3 has a key role in the regulation of fundamental cellular processes, such as cellular proliferation and differentiation death. Dkk3 acts in a variety of cancers as an oncosuppressor protein by inducing apoptotic death [19–22]; although, it might promote cancer cell spreading by inducing angiogenesis [23,24]. Dkk3 promotes kidney cell death [25] and, in contrast, promotes survival in retina cells [26]. The aim of this study was to assess how Dkk3 is involved in regulating the Wnt/ β -catenin pathway during instances of cerebral ischemia and reperfusion injury.

2. Materials and Methods

2.1. Animals

Male Wistar rats (200–250 g) were purchased from Envigo (Milan, Italy) and employed for this study. All animal experiments agree with the new regulations in Italy (D.Lgs 2014/26), EU regulations (EU Directive 2010/63).

2.2. Surgical Procedure

Animals were anesthetized and middle cerebral artery (MCA) occlusion was performed by introducing a 4.0 nylon monofilament, precoated with silicone via the external carotid artery into the internal carotid artery to occlude the MCA [27,28]. Control animals were subjected to the same procedure, but no filament was introduced. At the end of the surgery, anesthesia was discontinued, and the animals were returned to a prone position [29].

The experimental procedures were performed according to the following protocol:

- (1) After ischemia animals were sacrificed at different timepoints (1 h, 6 h, 12 h and 24 h) and brain tissues were harvested for further analysis (Figure 1). Experimental groups:
 - Control animals sacrificed after 1 h from the surgery.
 - MCAO animals sacrificed 1 h after the end of occlusion.
 - Control animals sacrificed after 6 h from the surgery.
 - MCAO animals sacrificed 6 h after the end of occlusion.
 - Control animals sacrificed after 12 h from the surgery.
 - MCAO animals sacrificed 12 h after the end of occlusion.
 - Control animals sacrificed after 24 h from the surgery.
 - MCAO animals sacrificed 24 h after the end of occlusion.

- (2) One and six h after ischemia, animals were administered with curcumin (100 mg/Kg) [30], and sacrificed 24 h after the end of the occlusion (Figure 2). Brain tissues were harvested for further analysis. Experimental groups:
- Control animals sacrificed after 24 h from the surgery,
 - MCAO animals sacrificed 24 h after the end of occlusion,
 - MCAO animals administered with curcumin 1 and 6 h after the end of the occlusion and sacrificed 24 h after the end of occlusion.

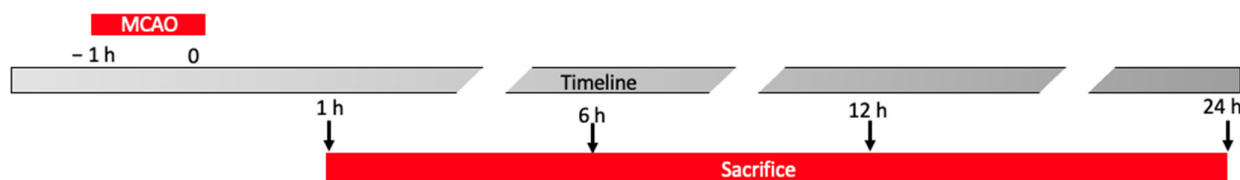


Figure 1. Experimental protocol 1.

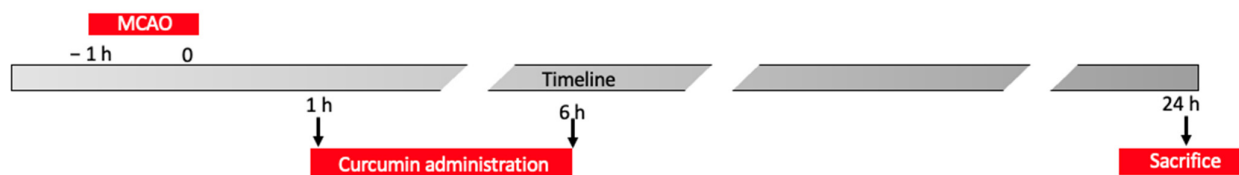


Figure 2. Experimental protocol 2.

2.3. Quantification of Infarct Volume

Tissues were incubated in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) for 30 min at 37 °C, processed, and quantified as previously described [31]. All analyses were carried out by two observers blinded to the treatment.

2.4. Western Blot Analysis

Western blots were performed as already described [32]. Brain samples were suspended in buffer A (0.2 mM phenylmethylsulfonyl fluoride, 0.15 μ M pepstatin A, 20 μ M leupeptin, and 1 mM sodium orthovanadate) homogenized for 2 min, and centrifuged at 10,000 \times g for 10 min at 4 °C. Supernatants represented the cytosolic fraction. The pellets, containing nuclei, were re-suspended in buffer B (150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 10 mM Tris-HCl pH 7.4, 0.2 mM phenylmethylsulfonyl fluoride, 20 μ M leupeptin, and 0.2 mM sodium orthovanadate). After centrifugation for 30 min at 15,000 \times g at 4 °C, the supernatants contained nuclear proteins. Samples were stored at -80 °C for further analysis. Filters were probed with the primary antibodies: anti-Dkk3 (ab2459), or anti-wnt3a (ab28472), or anti-Frz1 (ab126262), or anti-PIWI1a (ab12337), or anti- β -catenin (ab32572), or anti-FOXO1 (sc-376471), or anti-Caspase 3 (ab13847), or anti-Bax (Santa Cruz Biotechnology, Dallas, TX, USA, sc526), or anti-Bcl-2 (Santa Cruz Biotechnology, sc492), or anti-Ngn2 (Santa Cruz Biotechnology, sc-293430), or anti-Tbr2 (Santa Cruz Biotechnology, sc-376776), or anti-Pax6 (ab109233) at 4 °C overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat antirabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. Blots were also incubated with primary antibodies against β -actin protein (Sigma-Aldrich, St. Louis, MI, USA) or laminin (Sigma-Aldrich Corp.), which were used as internal standards.

2.5. Statistical Analysis

All values are shown as the mean \pm standard error of the mean (SEM) of N observations. Data were analyzed by one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. A *p*-value of less than 0.05 was considered significant: * *p* < 0.05

versus sham; ** $p < 0.01$ versus sham; *** $p < 0.001$ versus sham; # $p < 0.05$ versus MCAO; ## $p < 0.01$ versus MCAO; ### $p < 0.001$ versus MCAO.

3. Results

3.1. Analysis of Ischemic Brain Area

MCAO was performed in rats, sacrificing animals at different timepoints. TCC staining showed increased infarct volume with increased time of reperfusion (Figure S1). In order to evaluate the expression of Dkk3 in the brain at different time points and how it modulates the WNT/ β -catenin pathway, Western blot analysis was performed. Dkk3 expression strongly increased with the time of reperfusion reaching the highest level at 24 h (Figure 3A). Levels of Wnt3a (Figure 3B), Frz1 (Figure 3C) and PIWI1a (Figure 3D) decreased with the time of reperfusion as compared to the control. Nuclear expression of FOXM1 (Figure 3E) and β -catenin (Figure 3F) decreased in MCAO animals with the time of reperfusion as compared to the control. All investigated proteins reached the lowest level at 24 h of reperfusion.

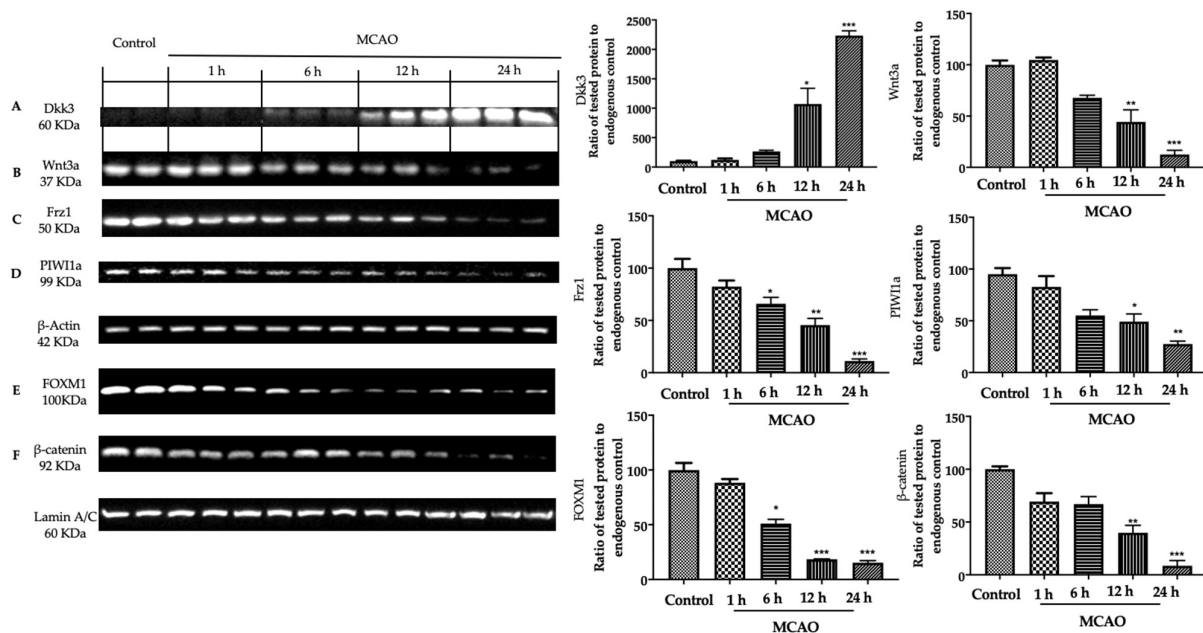


Figure 3. Analysis of Dkk3 levels in ischemic brain: Western blot analysis of (A) Dkk3, (B) Wnt3a, (C) Frz1, (D) PIWI1a, (E) FOXM1, (F) β -catenin. For these analyses, $n = 5$ animals for each group were employed. Data were analyzed by one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. A p -value of less than 0.05 was considered significant: * $p < 0.05$ versus sham; ** $p < 0.01$ versus sham; *** $p < 0.001$ versus sham.

3.2. Analysis of Apoptosis and Neurogenesis in Ischemic Brain

Western blot analysis showed increased Caspase 3 (Figure 4A) and Bax (Figure 4B) expression in MCAO animals with the increased time of reperfusion, as compared to control, while Bcl-2 (Figure 4C) expression decreased in animals subjected to MCAO with the time of reperfusion. Western blot analysis also showed decreased Tbr2 (Figure 4D), Ngn2 (Figure 4E) and Pax6 (Figure 4F) expression in animals subjected to MCAO at the different times of reperfusion. The lowest expression was reached at 24 h after the occlusion.

3.3. Analysis of Dkk3 Expression after Curcumin Administration in Ischemic Brain

TCC analysis showed decreased infarct volume in animals administered with curcumin, as compared to the MCAO group (Figure 5A). Western blot analysis showed decreased Dkk3 expression (Figure 5B) and increased Wnt3a (Figure 5C), Frz1 (Figure 5D) and PIWI1a (Figure 5E) in animals administered with curcumin, as compared to MCAO rats.

Nuclear FOXM1 (Figure 5F) and β -catenin (Figure 5G) expression increased in curcumin-administered rats, as compared to the MCAO group.

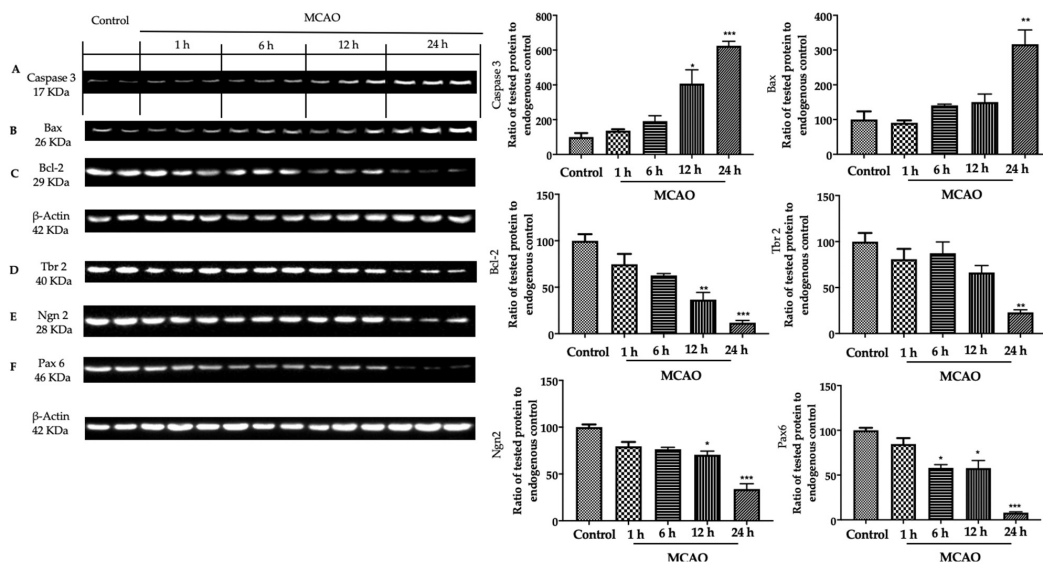


Figure 4. Analysis of apoptosis and neurogenesis in ischemic brain: Western blot analysis of (A) Caspase 3, (B) Bax, (C) Bcl-2, (D) Tbr 2, (E) Ngn 2, (F) Pax 6. For these analyses, $n = 5$ animals for each group were employed. Data were analyzed by one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. A p -value of less than 0.05 was considered significant: * $p < 0.05$ versus sham; ** $p < 0.01$ versus sham; *** $p < 0.001$ versus sham.

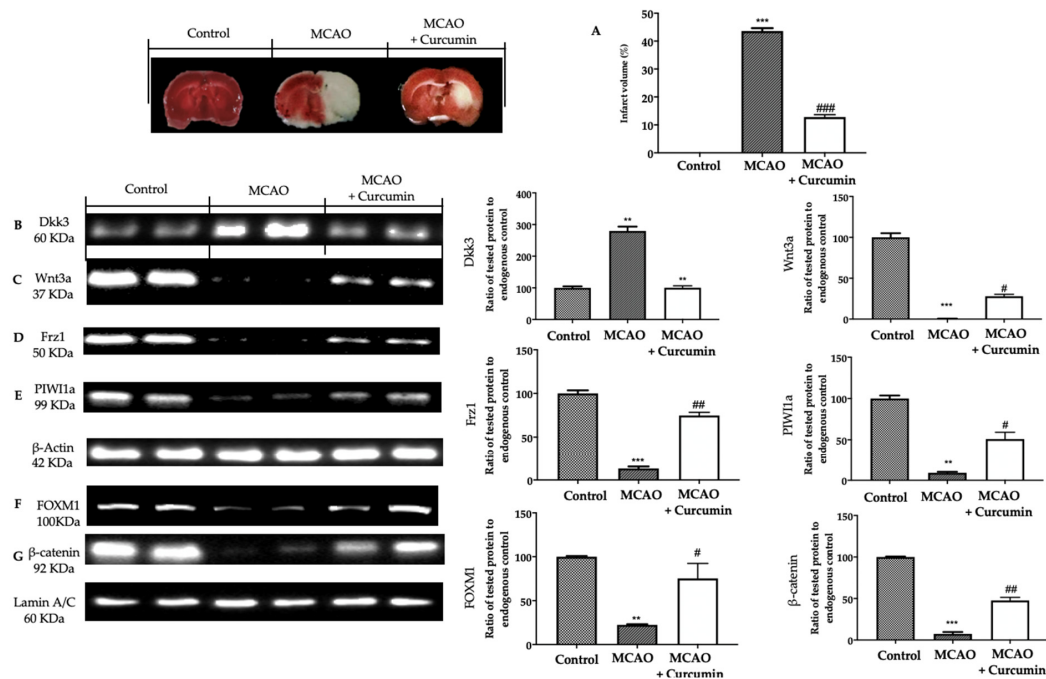


Figure 5. Analysis of Dkk3 in ischemic brain after curcumin administration: TCC analysis: (A) Infarct volume (%); Western blot analysis of (B) Dkk3, (C) Wnt3a, (D) Frz1, (E) PIW1a, (F) FOXM1, (G) β -catenin. For these analyses, $n = 5$ animals for each group were employed. Data were analyzed by one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. A p -value of less than 0.05 was considered significant: ** $p < 0.01$ versus sham; *** $p < 0.001$ versus sham; # $p < 0.05$ versus MCAO; ## $p < 0.01$ versus MCAO; ### $p < 0.001$ versus MCAO.

3.4. Analysis of Apoptosis and Neurogenesis after Curcumin Administration in Ischemic Brain

Western blot analysis showed increased Caspase 3 (Figure 6A) and Bax (Figure 6B) expression in MCAO animals, as compared to control, while Bcl-2 (Figure 6C) expression increased in animals subjected to MCAO. Curcumin administration decreased Caspase 3 (Figure 6A) and Bax (Figure 6B) levels and increased Bcl-2 (Figure 6C) expression. Western blot analysis also showed decreased Tbr 2 (Figure 6D), Ngn2 (Figure 6E) and Pax6 (Figure 6F) expression in animals subjected to MCAO, as compared to control. Curcumin administration increased Tbr 2 (Figure 6D), Ngn2 (Figure 6E) and Pax6 (Figure 6F) levels.

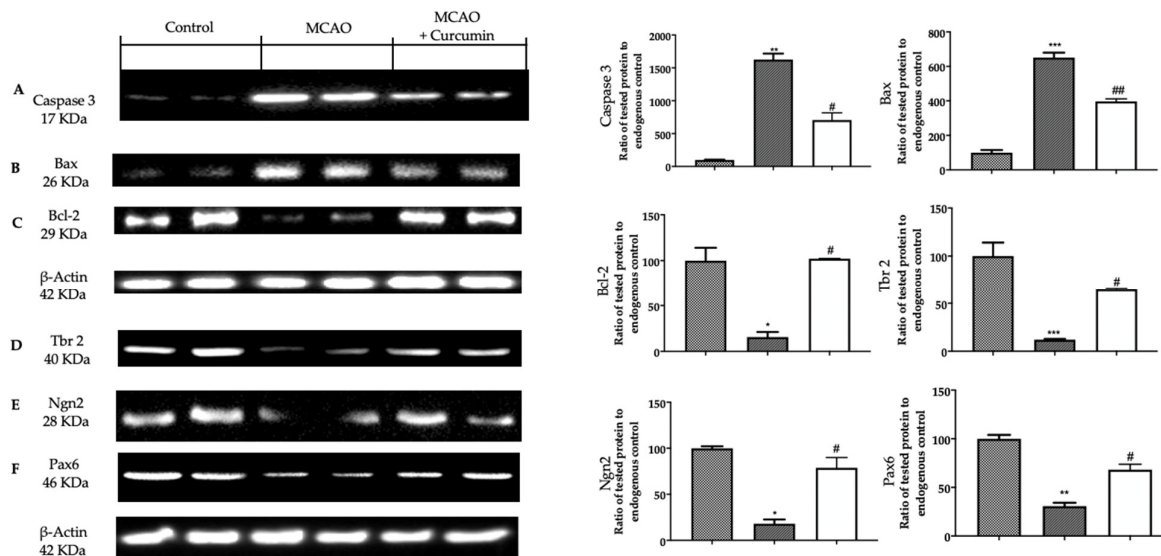


Figure 6. Analysis of apoptosis and neurogenesis in ischemic brain after curcumin administration: Western blot analysis of (A) Caspase 3, (B) Bax, (C) Bcl-2, (D) Tbr 2, (E) Ngn 2, (F) Pax 6. For these analyses, $n = 5$ animals for each group were employed. Data were analyzed by one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. A p -value of less than 0.05 was considered significant: * $p < 0.05$ versus sham; ** $p < 0.01$ versus sham; *** $p < 0.001$ versus sham; # $p < 0.05$ versus MCAO; ## $p < 0.01$ versus MCAO.

4. Discussion

Dkk3's role and function in the management of the Wnt pathway are still unclear even though it shares about 40% of protein identity with other well-characterized members of the Dkk family. Dkk3 diverges from all other family members because its modulatory action on this signaling is context-dependent and complex. The activation of the Wnt pathway has been described as neuroprotective in previous studies on cerebral ischemia and reperfusion [33]. This study focuses on the role of Dkk3 in Wnt signaling during neuronal injury. In the first part of our investigation, we investigated the expression of Dkk3 in the cerebral cortex surrounding the ischemic focus at different timepoints after ischemia. Dkk3 levels strongly increased with the time of reperfusion, reaching the highest expression at 24 h after ischemia. The upregulation of Dkk3 was accompanied by the down-regulation of Wnt3a, Frz-1 and PIWI1a expression at the same timepoints. Moreover, FOXM1 and β -catenin levels in nuclei were decreased. GSK3 β is one of the main effectors of the Wnt/ β -catenin pathway [34]. GSK3 β induces the phosphorylation of β -catenin inducing its degradation into the cytoplasm [35,36]. GSK3 β activity may be blocked by the phosphorylation of its Ser-9. This enzymatic inhibition leads to the stabilization of β -catenin. Once accumulated in the cytosol, β -catenin translocates into the nucleus and binds to the TCF/LEF factor, inducing the transcription of several genes [37]. In particular, the down-regulation of the Wnt/ β -catenin induced by MCAO increased apoptosis, increasing Bax and Caspase 3 expression and reducing Bcl-2 levels. Additionally, the down-regulation of the Wnt pathway by Dkk3 also induced the decreased expression

of the neurogenesis-promoting elements Tbr2, Ngn2 and Pax6. Ngn2 supported the differentiation of hippocampal neuroblasts [38]. Tbr2 supported the conversion from radial glia to progenitor cells [39], and Pax6 promotes the differentiation of neurons in the olfactory bulb [40]. All these data indicated that the cerebral ischemia and reperfusion injury increased the expression of Dkk3 in the borders of the ischemic focus leading to down-regulation of Wnt/ β -catenin signaling. To examine how the expression of Dkk3 changes in time during ischemic stroke and how it manages the Wnt/ β -catenin pathway, we decided to employ curcumin, a well-known activator of Wnt/ β -catenin signaling, to investigate its effect on Dkk3. In particular, curcumin was administered 1 and 6 h after ischemia, and animals were sacrificed 24 h later when the expression of Dkk3 was higher. Our results showed that curcumin administration decreased Dkk3 expression, which, in turn, increased Wnt3a, Frz1 and PIWI1a levels. Well in line with these data, curcumin administration increased nuclear β -catenin and FOXM1 expression. The down-regulation of Dkk3 by curcumin led to reduced apoptosis by decreasing Caspase 3 and Bax expression and increasing Bcl-2 levels. Indeed, curcumin promotes neurogenesis as shown by Tbr2, Ngn2 and Pax6 increased levels.

However, this research has some limitations. Limitations of the model include its end-arterial occlusive nature that makes the lesion resistant to flow enhancement strategies. Additionally, deep investigations are required to describe how Dkk3 would influence other molecular pathways involved in the disease.

Overall, our results showed that Dkk3 acts as an inhibitor of Wnt/ β -catenin signaling during cerebral ischemia. Additionally, its inhibition and the contextual activation of the Wnt/ β -catenin pathway would be protective against ischemic stroke.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/biomedicines11030815/s1>, Figure S1: Analysis of ischemic brain area.

Author Contributions: Conceptualization, M.C.; Formal analysis, V.B.; Funding acquisition, S.C.; Methodology, R.F.; Project administration, R.D.P.; Software, R.S.; Supervision, A.F.G.; Validation, G.C.; Writing—review and editing, S.M.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study was approved by the University of Messina Review Board for the care of animals. Animal care conformed to Italian regulations on the use of animals for experimental and scientific purposes (D.Lgs 2014/26 and EU Directive 2010/63).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest: The authors declare no conflict of interest.

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