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Tuhfatul Ulya, Chrismawan Ardianto*, Putri Anggreini, Aniek Setiya Budiatin, Dwi Setyawan and Junaidi Khotib

Quercetin promotes behavioral recovery and biomolecular changes of melanocortin-4 receptor in mice with ischemic stroke

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Abstract

Objectives: Ischemic stroke is known as a common causes of disability, lower psychological well-being as well as preventable death. The pathogenesis of ischemic stroke process becomes worse immediately after oxidative stress occurs. One of the flavonoids with antioxidant abilities is quercetin. This study was aimed to investigate quercetin administration on the behavioral functions (motor and sensory) and expression of melanocortin-4 receptor (MC4R) in mice with ischemic stroke.

Methods: Male ICR mice were divided into sham, stroke, stroke with quercetin 100, 150, and 200 mg/kg. The stroke model was performed by blocking the left common carotid artery for 2 h. Quercetin was intraperitoneally administered daily for seven days. Evaluation was conducted during two weeks after induction using ladder rung walking test and narrow beam test for motoric function and adhesive removal tape test for sensory function. On day-14 mice were sacrificed, MC4R expression in the dorsal striatum was determined using RT-PCR.

Results: Stroke decreased the motor, sensory function and MC4R mRNA expression in dorsal striatum. Quercetin improved motor and sensory function, and upregulated expression of MC4R.

Conclusions: Quercetin administration after ischemic stroke improves behavioral function, possibly through the upregulation of MC4R in the brain.

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Keywords: dorsal striatum; ischemic stroke; melanocortin-4 receptor; motor; preventable death; quercetin.

Introduction

Ischemic stroke is a major risk factor for disability and the third leading cause of death all worldwide [1]. The rapid oxidation of protein, nucleic acid, and lipid is very worrying in the brain. The pathogenesis process triggers signals for interrelated metabolic processes, specifically decreased ATP production, increased intracellular calcium, and the formation of free radicals end up with neuronal cell death [2]. The condition becomes worse immediately after oxidative stress occurs, followed by the production of surplus reactive oxygen species (ROS) [3].

Oxidative stress is defined as an imbalance between ROS and their quenching by an antioxidant system, due to the overproduction of ROS and lack of antidotes [4]. Lots of evidence has shown that oxidative stress is one of the pathophysiology of early and primary cell death in the pathogenesis of ischemic stroke [2]. Several antioxidants were examined to enhance the defense system against nerve cell degeneration in order to prevent the progression of the harmful mechanisms involved in ischemia [5, 6].

Flavonoids are plant secondary metabolites that have been widely studied. The flavonoid group has a chemical structure that plays a role in its activity as antioxidants [7]. Quercetin is a flavonoid with antioxidant activity due to the presence of catechol groups and OH groups, the antioxidant pharmacophores [8–10]. In cerebral ischemia, the melanocortin system has neuroprotective effects likely mediated by the melanocortin-4 receptor (MC4R). Stimulation of MC4R affords neuroprotection by counteracting inflammation process and apoptosis [11]. However, there is no evidence showing the quercetin effect on the ischemic stroke-induced motor and sensory impairment from day to day, and its correlation with MC4R expression in the dorsal striatum.

The dorsal striatum is known as an important brain area in motor regulation. Previous study showed that injury to the striatum affects the recovery of gait in stroke

patients [12, 13]. Therefore, this study aimed to investigate the effects of quercetin administration as an antioxidant and neuroprotective agent on improving motor and sensory function and biomolecular change (MC4R expression) in dorsal striatum mice with ischemic stroke. This study used a left unilateral common carotid artery occlusion model to induce stroke in mice resulting in hypoxia on the brain tissue [14].

Materials and methods

Animals

Male ICR mice, weighing between 25 and 30 g were used. All mice were treated at a temperature of 25 °C ± 2 °C with a humidity of 60 ± 10%, free access to food and water was also provided. All experiments were carried out at the Animal Research Laboratory of the Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia. The experimental protocol was approved by the Animal Care and Use Committee (ACUC), Faculty of Veterinary, Universitas Airlangga.

Drug and stroke surgery

Quercetin (Tokyo Chemical Industry, Tokyo, Japan) was dispersed into a mixture of carboxymethyl cellulose and tween 80 (1:1), with preparation less than 30 min before injection.

After acclimatization, the mice were randomly divided into five groups (n=8 in each group): (1) Sham group, (2) stroke group, (3) stroke with quercetin 100 mg/kg, (4) 150 mg/kg, (5) 200 mg/kg. Xylazine and ketamine were administered for anesthesia. The animals are positioned supine on the surgical table. Around 1 cm incision in the neck midline was made. The left common carotid artery was exposed and blocked by surgical silk for 2 h and then released for reperfusion. The Sham group was subjected to the same procedure without a carotid block. Quercetin-treated groups received a intraperitoneal injection of quercetin 30 min after stroke surgery, another group received an intraperitoneal injection of tween 0.5%. Quercetin was administered once a day for seven days intraperitoneally.

Ladder rung walking test

The protocol, shown in Figure 1, was performed in accordance to the previous study [15] by using the ladder rung walking test apparatus shown in Figure 1, on day 0 (before stroke surgery), 1, 4, 7, 10, 14 after stroke induction. All mice were allowed to walk through the cylindrical stairs arranged with varying distances in 1-m course. Movement of the right mice hind limb in each step was observed. The camera was used for record it. Mice that slip show a decrease or impaired motor function. A seven-category scale was assessed according to the position of the foot placement on the rungs and the shape of error [16]. All animals were trained and tested five times per-session. Then, the average error score was analyzed. Error score data was presented as a percent of 100%.

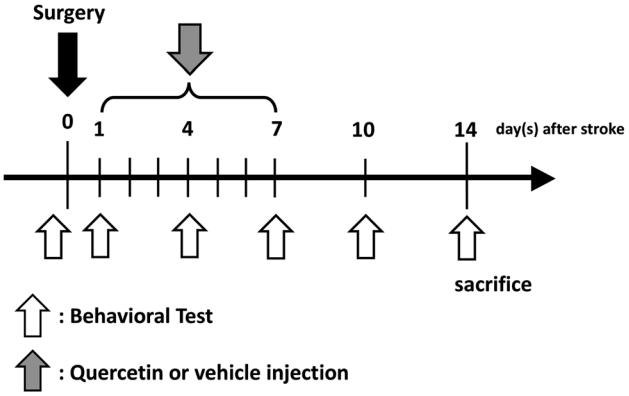

Figure 1: Schematic illustration of experimental design for examining the effect of quercetin on behavioral and biomolecular changes after ischemic stroke.

Narrow beam test

The protocol was performed following the previous study [16]. Mice were placed to cross the narrow beam apparatus. Mice were placed in the initial zone, and the stopwatch started immediately after the animal was released. The time was recorded when the animal starts walking past the start line and get a slip. The time taken by animals to reach the final platform at the end of the beam was also recorded, with a maximum time of 15 s. Both the time until mice are found to slip or fall, as well as the total time taken to cross the beam, were measured for five trials. The tests performed on day 0 (before stroke surgery), 1, 4, 7, 10, 14 (after stroke surgery).

Adhesive removal tape test

The protocol was performed following the previous study [17] with a few modifications. In this test, adhesive tapes of the same size (1 × 1 cm) is placed on the right forelimb of each animal with equal pressure. Each group was randomized to the order of adhesive placement (right or left). The initial contact time by left forelimb called time to touch, and the time required for the mice to remove the tape called time to remove in a maximum of 120 s was recorded. This test was performed regularly on day 0 (before stroke surgery) and days 1, 4, 7, 10, 14 (after stroke surgery).

Reverse Transcription - Polymerase Chain Reaction (RT-PCR) Analysis

On day 14, the brain was extracted, the dorsal striatum was dissected and liquid nitrogen was used to immediately freeze the sample. Storage was carried out at −80 °C until use. RT-PCR was performed according to Ardianto et al. [18]. The PureLink® RNA mini kit (Invitrogen, Cat No. 12183018A) was used to isolate the total RNA. GoScript ™ RT (Promega, Cat No. A5000) was used for reverse transcription. The following primers were used: MC4R (forward: 5′-TTA ATA CCT GCT AGA CAA CTC A-3′, reverse: 5′-ATG TAT ACT TCC CTC CAC CTC TG-3′), and β-actin (forward: 5′-ACCCACACTGTGCCCATCTAA-3′, reverse: 5′-GCCACAGGATTCCATTA CCCAA-3′). Amplification was conducted using thermal cycler (Applied

Biosystems, USA) for RT-PCR with total cycle of 33. The denaturation cycle was carried out at 94 °C for 30 s, followed by the annealing at 58 °C for 1 min, and the extension at 72 °C for 1 min. Electrophoresis (MSMIDI Duo, USA) was used to analyze PCR products. Ethidium bromide (Sigma-Aldrich) was used to dye 2% Agarose gel. The band was captured with the aid of UV transillumination. ImageJ software (NIH, MD, USA) was used for image analysis.

Statistical analysis

Data were presented as mean values ± SEM. Behavioral test and body weight data were analyzed using a two-way ANOVA followed by the Bonferroni post-hoc test. Relative expression of MC4R was analyzed using a one-way ANOVA followed by the Bonferroni post-hoc test. The difference was considered significant if $p<0.05$ (95%).

Results

The effect of quercetin on body weight

Animal body weight was measured to determine the effect of quercetin administration and stroke induction on nutritional deficits and metabolic disorders that might occur. The results of weight measurement showed that there were no significant differences between the study groups (Figure 2). In addition, there were no significant changes between giving quercetin at various doses to animal body weight.

The effect of quercetin on ladder rung walking test

Stroke group with vehicle administration showed a significant decrease in motor function compared to the Sham group (two way ANOVA, stroke, $F_{1,14}=38.59$, $p<0.001$ vs. Sham) starting from day 1 through 14. In contrast, the stroke group treated with quercetin at doses of 100, 150, and 200 mg/kg showed a significant improvement in motor function when compared to the stroke group with vehicles, starting from day 1 and gradually increasing on days 4 through 14 (Figures 3 and 4).

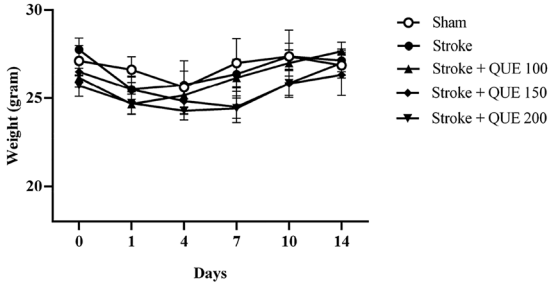

Figure 2: The effect of quercetin on body weight of mice induced ischemic stroke (mean ± SEM) of 6–8 mice. Administration of quercetin were conducted repeatedly day 1–7. QUE, quercetin.

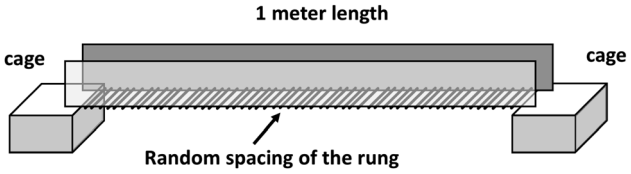

Figure 3: Ladder rung walking test apparatus for ladder rung walking test measurement.

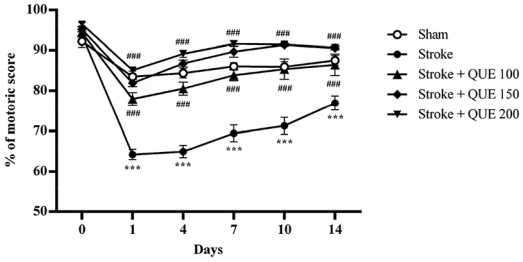

Figure 4: The motor function of ischemic stroke mice measured by ladder rung walking test (mean ± SEM) of 6–8 mice. ***p<0.001 vs. Sham group. ###p<0.001 vs. stroke group. QUE, quercetin.

The effect of quercetin on narrow beam test

In addition, we also examined the effect of quercetin on stroke induction on motor function improvement using the narrow beam test. The results of the latency to slip showed that the stroke group had a significantly lower motor function compared to the Sham group on day 4 (Figure 5A). This was evidenced by mice that slips faster than the Sham group. Furthermore, the stroke group showed a decrease in motor function by spent more time to reach the finish, the difference was significant compared to the Sham group on day 4 (Figure 5B).

In contrast, the stroke group with quercetin administration (200 mg/kg) showed significantly improved motor function by increased time latency to slip on day 4. Then, the stroke group with quercetin at a dose of 150 and 200 mg/kg significantly suppressed time to reach the finish compared with the stroke group starting on day 1 (Figure 5).

The effect of quercetin on adhesive removal tape test

The effects of stroke induction and quercetin administration on the sensory functions of mice showed the stroke

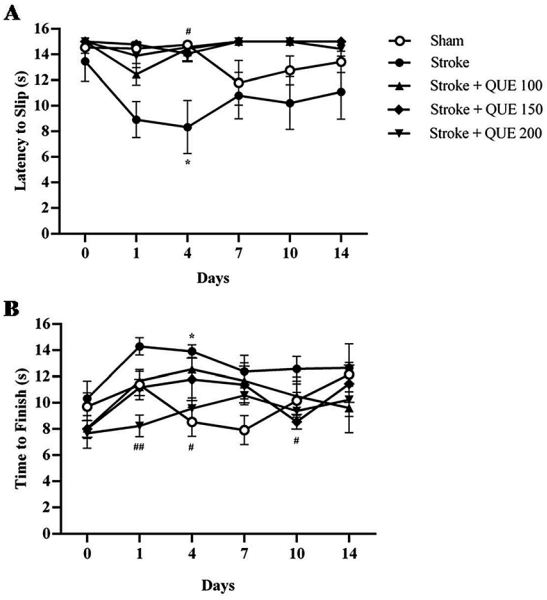

Figure 5: The effect of quercetin on motor function of ischemic stroke mice. (A) Latency to slip and (B) time to finish the beam were measured by narrow beam test (mean ± SEM) of 6–8 mice. *p<0.05 vs. Sham group. #p<0.05, ##p<0.01 vs. stroke group. QUE, quercetin.

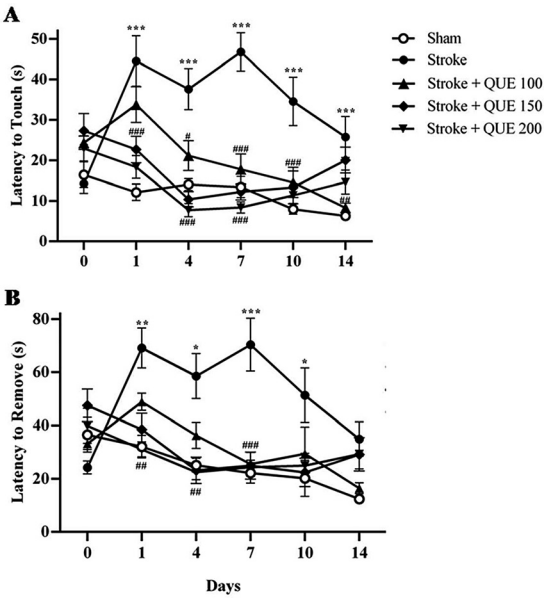

Figure 6: The effect of quercetin on sensory functions of mice induced by ischemic stroke. (A) Latency to touch the tape and (B) latency to remove the tape were measured by adhesive removal tape test (mean ± SEM) of 6–8 mice. *p<0.05, **p<0.01, ***p<0.001 vs. Sham group. #p<0.05, ##p<0.01, ###p<0.001 vs. stroke group. QUE, quercetin.

group had a significantly lower function than the Sham group on the latency to touch (Figure 6A), and latency to remove (Figure 6B) values starting on day 1, followed by day 4 through 14.

Furthermore, administration of quercetin (150 and 200 mg/kg) was significantly improves sensory function compared to the stroke group in the latency to touch from day 1, whereas doses 100 mg/kg from day 4 through 14. Then, administration of quercetin doses of 150 and 200 mg/kg significantly suppressed the latency to remove time compared to the stroke group starting on day 1, whereas doses 100 mg/kg from day 7 (Figure 6).

The effect of quercetin on melanocortin-4 receptors

The results of examination MC4R expression in the dorsal striatum of the brain showed a significantly lower MC4R expression on stroke group compared to the Sham group (one way ANOVA, stroke, $F_{2,6}$=357.6, p<0.01 vs. Sham). In contrast, the stroke group with 100 mg/kg quercetin administration significantly increased MC4R expression compared to the stroke group (p<0.001 vs. stroke) on days 14 (Figure 7).

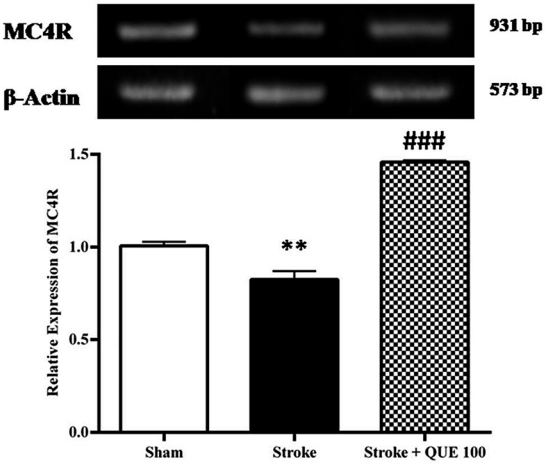

Figure 7: The effect of quercetin on relative expression of MC4R in dorsal striatum of mice induced by ischemic stroke (mean ± SEM) of three mice. **p<0.01 vs. Sham group. ###p<0.001 vs. stroke group. Bp, base pair; MC4R, melanocortin-4 receptor; QUE, quercetin.

Discussion

This study was designed to determine changes of behavioral function (motor and sensory) and biomolecular changes in MC4R expression in the brain of mice after

stroke induced by the left unilateral common carotid artery occlusion method, and the improvements due to quercetin treatment. This stroke induction method is known to cause impairment of motor and sensory function and lead to neuronal cell death in the brain [14].

Quercetin administration did not affect mice's body weight from day 1 to day 14. This result is in agreement with studies from Barrenexte et al. [19] that dietary foods containing 0.5% quercetin for 28 days did not affect the body weight of mice compared to the control group. Similar results were shown by a high-sucrose diet containing 0.07, 0.2, and 0.02% quercetin for four weeks, which does not affect the body weight of rats [20].

The motor function examination results using the ladder rung walking test method showed a significant attenuation in motor function impairment after quercetin administration in days 1, 4, 7, 10, and 14 compared to the stroke group. Giving quercetin at a dose of 100, 150, and 200 mg/kg improves motor function in ischemic stroke conditions. Similarly, the results of the motor function test using the narrow beam test method also showed notable improvement with quercetin administration as compared to the stroke group. The result showed a dose-dependent trend of the effect of quercetin to improve motor function. A previous study reported that the post-injury administration of quercetin (50 mg/kg) improves the motor function between day 1 until 5 after trauma compared with the traumatic brain injury group [21]. Together, these results suggest that quercetin attenuates the stroke-induced motor function deficits.

Furthermore, our present data show stroke-induced sensory impairment that did not recover in a two-week examination period. Moreover, the present study revealed that quercetin (100, 150, and 200 mg/kg) decreased the time to touch and to remove the adhesive tape in ischemic stroke mice, starting from day 1 after stroke induction. A study from Chen et al. [22] showed that quercetin administration accelerates full sensory recovery in 18 days in mice with sciatic nerve-crush injury compared to the untreated mice. Together with our result, it is suggested that quercetin attenuates the sensory function impairment after an ischemic stroke attack. Furthermore, we found that all doses (100, 150, and 200 mg/kg) effectively improve stroke related-behaviors. Each dose has a certain level of speed in improving motor and sensory function. Although higher doses showed faster improvement, the three doses were not significantly different, meaning they were equally effective in improving motor and sensory function due to stroke in a relatively fast time.

It is well known that melanocortin system activity is involved in responding to the neuroinflammatory process in the brain. The activation, as well as the upregulation of MC4R, has been the biomolecular marker representing the anti-inflammatory process [23]. MC4R is reported expressed in the dorsal striatum, one of the critical brain regions that regulate motor function [24]. Therefore, we investigate the expression of MC4R in the dorsal striatum and effect of quercetin. Since we already found that 100, 150 and 200 mg/kg dose of quercetin are effective in ameliorates the stroke related-behaviors, for MC4R expression analysis we used representative yet effective dose of quercetin which is 100 mg/kg. Our present study revealed that the MC4R mRNA level in the dorsal striatum decreased in stroke. Furthermore, we found that treatment with quercetin ameliorated the decrease MC4R mRNA level in the dorsal striatum. A previous study showing that MC4R mRNA was upregulated in the striatum after severe hypoxic-ischemic brain injury compared with control [25]. The present result suggests that the upregulated MC4R mRNA in the dorsal striatum may reflect the brain capability to reduce the inflammatory process and restore the deficit in brain function affected by an ischemic stroke.

Previous study showed that MC4R stimulation prevents mitochondrial stress and oxidative damage due to ethanol through the activation of the Nrf-2 pathway in neuron culture [26]. In addition, quercetin has been reported to modulate the activation of the Nrf-2-ARE pathway [27]. Nrf2-ARE pathway activation increases the expression of detoxification enzymes such as glutamate-cysteine ligase and synthesis of glutathione, an endogenous antioxidant [28]. In conclusion, our present results indicate that quercetin attenuates the motor and sensory deficits induced by an ischemic stroke in mice. Additionally, it is suggested that quercetin reverse the functional impairment through the upregulation of MC4R in the dorsal striatum. Further study is needed to clarify the direct evidence explaining how the quercetin affects the expression of MC4R.

Conclusions

This study shows that quercetin ameliorates motor and sensory impairment due to ischemic stroke conditions. Further, it is suggested that quercetin may promotes functional recovery from ischemic stroke condition by the MC4R upregulation.

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Ethical approval: All experiments were performed at the Animal Research Laboratory of the Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia in accordance with the Guide for care and use of laboratory animal issued by National Institution of Health revised in 1985. The experimental protocol was approved with letter No: 2.KE.111.07.2019 by the Animal Care and Use Committee (ACUC), Faculty of Veterinary, Universitas Airlangga.

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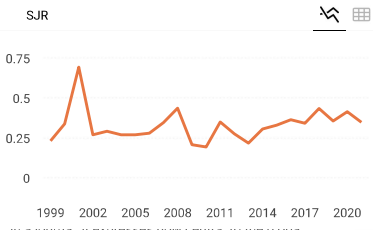

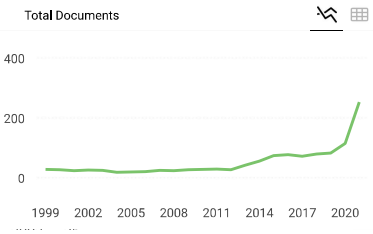

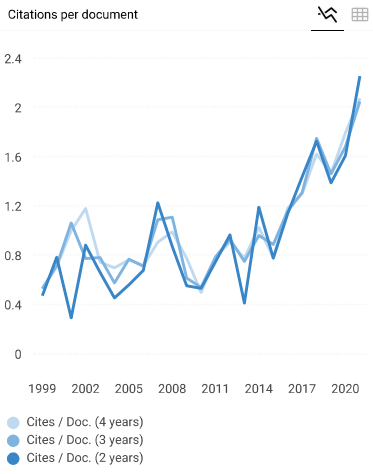

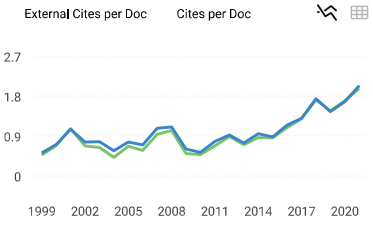

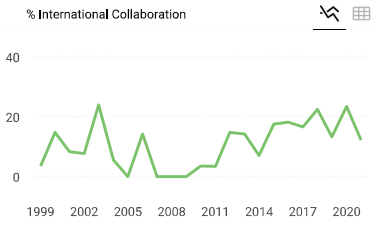

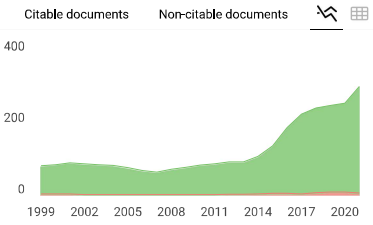

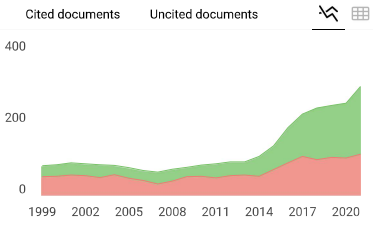

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