International Journal of Scientific Advances
ISSN: 2708-7972
Volume: 5 | Issue: 6 | Nov – Dec 2024 Available Online: www.ijscia.com
DOI: 10.51542/ijscia.v5i6.67

Histopathology of Interstitial Fibrosis Degree Following Administration of East Java Nano Propolis in Adenine-Induced Chronic Kidney Disease Model (*Rattus norvegicus*)

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ABSTRACT

Introduction: Chronic kidney disease is a worldwide health concern, with chronic inflammation and fibrosis formation as its key pathological features. Current interventions for chronic kidney disease show substantial side effects and a high cost that poses a social and economic burden. Propolis is a natural product from bees that exhibits anti-inflammatory and antioxidant effects, making it a potential therapy option. ***Objective:*** This experimental study emphasizes the relationship between varying dosages of propolis and interstitial fibrosis degree in chronic kidney disease models. ***Method:*** A true experimental post-test-only controlled group study to asses east java nano propolis effects regarding interstitial fibrosis degree in adenine-induced chronic kidney disease model. The rats are divided into four groups: Group K0 (5 rats without daily nano propolis administration), group P1 (5 rats with 100 mg/kg WB/day intervention), group P2 (6 rats with 200 mg/kg WB/day intervention), and group P3 (5 rats with 300 mg/kg WB/day intervention). ***Result:*** Group P2 exhibits a significant difference towards K0 (p=0.045), and Group P1 and P2 do not exhibit a significant difference (p=0.848; 0.120). ***Conclusion:*** Propolis can significantly reduce the degree of interstitial fibrosis in the adenine-induced chronic kidney disease model compared to a control group.

Keywords: propolis; chronic kidney disease; animal model; adenine.

INTRODUCTION

Chronic kidney disease (CKD) is a worldwide health problem with a prevalence estimated in the range of 10% of all people in the world. It is estimated that CKD is now one of the leading causes of mortality and morbidity in the world and has earned its place as a global burden. Studies in 2017 show that approximately 850 million people are now experiencing CKD and that number is expected to increase in the following years [1]. It is widely known that CKD is associated with other common morbidity such as diabetes mellitus and hypertension with oxidative phosphorylation and inflammation as its key pathophysiology [2]. Clinical diagnosis of CKD uses biochemical parameters, being their estimated glomerular filtration rate (eGFR). The general consensus stated that eGFR < 60 ml/min/1.73 m2 is considered to be CKD though it may vary around the world [3]. Treatment options for CKD patients are divided into 2 groups, one being conservative and the other being renal replacement therapy (RRT) both being derived from each patient's eGFR. The main goal of CKD treatment is not to cure the disease itself but rather to preserve current kidney function [4].

Hemodialysis is part of RRT and it is prescribed for patients in later stages of CKD. Hemodialysis is not a perfect treatment with its fair share of adverse effects such as hemolysis and dialysis equilibrium syndrome among others [5]. Mortality rates for such patients are high. The mortality rate of hemodialysis patients is 15% and up to 50% in patients that use hemodialysis for longer than 5 years [6]. On the other hand, conservative treatment plans are treating patient-specific underlying problems such as diabetes and hypertension as well as treating the main pathophysiology of CKD, which is chronic inflammation using strong anti-inflammatory drugs such as steroids is infamously known for their

adverse effects from long-term usage, known as steroid-associated adverse events [7]. This opens new urgencies and opportunities for treating CKD patients using other means of treatment that are not only cheaper but also pose a lower risk of adverse effects such as propolis.

Propolis is a natural product derived from bee's nests that have been extracted usually using water or alcohol. The bees collected propolis from the surrounding area of their nest, mainly leaves, flowers, and tree resins, that would be mixed with their gastrointestinal enzyme to create a mixture that they use as a glue for strengthening their nests. Because of the nature of propolis is collected by bees and is derived from their surrounding geographical and floral factors, the composition of propolis would be different from one region to another [8,9,10]. Though the exact composition of propolis from region to region is different, their main constituents are rather the same. According to studies done on stingless bees (Meliponini tribe) and honeybees (Apis mellifera), the organic component of propolis is only about 5% but it holds important constituents such as flavonoid, polyphenols, and many different types of acids such as cinnamic acid, fatty acid, carboxylic acid, and caffeic acid. Polyphenols in propolis, more specifically flavonoids, contain biologically active components namely galangin, pinocembrin, and chrysin [11].

Studies regarding the pharmacological effects of propolis depict various pharmacological effects such as but not limited to, anti-inflammatory, antioxidant, immunomodulator, and anticancer [12]. Their anti-inflammatory and antioxidant properties are thanks to their bioactive component such as flavonoid and caffeic acid. Caffeic acid contributes as its main anti-inflammatory constituent. This is due to its ability to reduce pro-inflammatory cytokines. On the other hand, flavonoids such as galangin, pinocembrin, and chrysin are responsible for their antioxidant effect by reducing the reactive oxygen species (ROS) and improving enzymatic activity regarding antioxidant properties via the Nuclear factor erythroid 2-related factor 2 (Nrf2) pathway [13,14].

CKD animal models are an openly discussed subject that uses various methods. Adenine-induced CKD models are widely used with a dosage ranging from 0.25% to 0.75% within a span of up to 16 weeks, which is approximately equal to 8 years of progression in humans. Recent studies elicit that dosages ranging from 0.25% to 0.5% for a span of a minimum of 3 weeks are recommended due to its nature of a similar progression rate as in humans [15]. Adenine is chosen because it could mimic a key pathophysiology in human CKD which is inflammation, by depositing crystals in the tubulointerstitial area in the kidney [16].

METHOD

This is a true experimental post-test-only controlled group study. The study aims to better understand the effects of nano propolis extract against the degree of interstitial fibrosis in the *Rattus norvegicus* CKD animal model. The rats are divided into four groups with one control group and three treatment groups. Both control and treatment groups are given food laced with adenine with a dosage of 0.25% for four weeks after acclimation for about a week. Upon its completion, one of the rats would be randomly selected from each group to confirm whether the adenine-laced diet was successful in making CKD animal models. Following its confirmation, the treatment groups were given their respective dose of nano propolis extract via nasogastric admission for two weeks. The dosage for the treatment group varies from 100 mg/kg BW/day for group P1, 200 mg/kg BW/day for group P2, and 300 mg/kg BW/day for group P3. Every group is also given a normal food and water diet. Control group K0 was not exposed to any treatment after their respective adenine diet. The sample size for each group is 5 for P1, P3, and K0, and 6 for P2. Each subject is about ten weeks old upon any intervention and at weighs about 150 milligrams.

The rats are closely monitored in an animal lab located in the Department of Biochemistry at Airlangga University in Surabaya, Indonesia in a controlled environment with a temperature of 25 degrees Celsius. Weekly weighing is conducted every Friday for evaluation. After two weeks of treatment, all rats from every group were terminated and all the organs were harvested, including both kidneys. The kidney samples were later transferred to a histology lab under the Department of Anatomy, Histology, and Pharmacology at Airlangga University where they would be stained with the Mallory-Azan method and observed under a microscope. Every kidney was photographed using the CellSense application with a minimum of ten fields of vision at a magnification of 200 times and saved in .tiff format. The photographs were transferred to the ImageJ program for debris clearing and histomorphometry using the thresholding method. The acquired data were relocated to Microsoft Excel and International Business Machine Corporation (IBM) Statistical Package for the Social Sciences (SPSS) version 23.0 for analysis. Shapiro-Wilk normality test was conducted, followed by Analysis of variance (ANOVA) and Tukey Honestly Significant Difference (HSD).

RESULT

21 CKD animal models that met the inclusion criteria were observed. After the Shapiro-Wilk normality test shows a normal distribution, one-way ANOVA and post hoc Tukey HSD were conducted. Their results according to each group are shown in Table 1.

TABLE 1: Degree of Interstitial Fibrosis in Each Group.

Group	n	Mean ± Std. Deviation	Sig. (ANOVA)
K0	5	34,76640 ± 11,027090	p = 0.035
P1	5	27,56900 ± 23,968849	
P2	6	10,21450 ± 6,893934*	
P3	5	13,68680 ± 8,978673	

Asterisk symbol represents a significant change in the control group (K0). The analysis showed that the treatment group P2 had a significant change towards group K0 (p=0.045), while the other treatment group such as P1 (p=0.848) and P3 (p=0.120) did not show a significant difference compared to group K0.

DISCUSSION

CKD is defined as a condition where there is a reduction in GFR with or without symptoms for at least 3 months with or without renal damage [17]. Renal damage is caused due to chronic inflammation because of the activation of the immune system such as macrophage activation that leads to the release of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6. The release of pro-inflammatory cytokine would bolster the translocation of NF-κB would play a role in the production of nitric oxide synthase, and later nitric oxide (NO), which would cause tissue damage as well as inflammation and feeling of pain [13]. Previous studies found that inflammation and oxidative stress are closely related. Moreover, chronic inflammation is known for its ability to develop scarring or fibrosis over time [18-20]. Renal fibrosis is a prominent finding in CKD patients because of extracellular matrix (ECM) deposition as a result of the activation of the main modulator for fibrosis in mammals called TGF-β cytokine. In normal conditions, TGF-β is well regulated by the body and fibrosis would not occur at a massive scale. However, due to certain long-term conditions such as chronic inflammation, TGF-β would mediate the deposition of ECM in an abnormal and unregulated amount, causing large-scale fibrosis in multiple organs including the kidney [21,22]. TGF-β is not only related to inflammation but also is related to reactive oxygen species (ROS). Upon the increase in the number of ROS due to a plethora of reasons such as the increase of NO and pro-inflammatory cytokine, ROS in return would increase the production and the activation of TGF-β. Interestingly, the growth of TGF-β would also increase the number of ROS. In other words, inflammation, oxidative stress, and fibrosis mechanisms are closely related [23,24].

Propolis is a product from bees collected from flowers and tree barks around their nest with the purpose of strengthening their nests. In addition, propolis is also pharmacologically proven to have several benefits including anti-inflammatory and antioxidant properties. This is a result of their bioactive components such as flavonoids and caffeic acid [8-11]. The bioavailability of propolis is low. This is due to its composition consisting mainly of lipids and wax [25]. For that reason, the usage of propolis in recent times has been improved by using nanotechnology in hopes of elevating its absorption. Thus, improving its result to be more uniform [26]. As mentioned before, renal fibrosis is the result of chronic inflammation and oxidative stress as a key pathophysiological feature of CKD and it is related to one another [2,18,19,24]. By reducing inflammation and oxidative stress, it would also reduce the degree of fibrosis when compared to the untreated group [25].

White strain of Winstar rats (*Rattus norvegicus*) is generally an acceptable model for kidney study. This is due to their similar physiology, and structure, and it also has a high reproducibility. Furthermore, this model could also replicate CKD at a faster rate. [27]. Adenine is a base purine that is metabolized as uric acid by xanthine oxidase in the blood. Uric acid is hard to reabsorb and it would leave 2,8-dihydroxyadenine crystals in the tubulointerstitial area in the kidney and would agitate immune reaction such as inflammation. This pathophysiological feature serves as the baseline for the usage of adenine for CKD animal models [15,28]. The dosage of adenine for CKD animals has changed over time. Previous studies that use adenine with a dosage of 0.75% prove to be more nephrotoxic than usual. More recent studies utilize dosages ranging from 0.25% to 0.5% and have proven to better mimic CKD progressivity. Time of intervention ranges from 3 to 16 weeks which translates to an age of 1.5 to 8 human years [15,29].

The result of this study shows a significant change in group P2 with a dose of 200 mg/kg WB/day (p=0.045). Previous systematic reviews regarding the effects of propolis in CKD animal models show similar histopathological findings in the reduction of renal fibrosis [25]. Similarly, another study using Taiwanese propolis with a dosage of 200 mg/kg WB/day for 16 weeks shows similar improvements toward tubulointerstitial fibrosis [30,31]. Other treatment groups however such as group P1 with a dose of 100 mg/kg WB/day and P3 with a dose of 300 mg/kg WB/day do not depict a significant change towards K0 with a p-value of 0.848 for P1 and 0.120 for P3 (p>0.05). Previous studies with dosages ranging from 50 mg/kg WB/day to 100 mg/kg WB/day do show a significant reduction in pro-inflammatory cytokines whilst reducing oxidative stress, but those dosage ranges do not indicate a significant histopathological finding [25].

Group P3 is rather an interesting case. As previously stated, polyphenols like flavonoids contribute an antioxidant constituent of propolis. However, polyphenols are not strictly found in propolis and are commonly found in plants, fruits, and other plant-based foods. Evidently, flavonoids have not only antioxidant but also anti-inflammatory properties at lower to moderate doses. At higher doses, however, in vitro studies have shown that polyphenols have the tendency to be pro-oxidative. This is because of the nature of polyphenols being auto-oxidative at higher dosages by binding to transition metal ions such as Cu, Zn, and Fe, as well as the promotion of lipid peroxidation product synthesis. This phenomenon supports the production of ROS such as superoxide anion radicals, thus creating a pro-oxidative environment. This is also true with caffeic acid and it would explain the results of P3 that have a higher dosage but have worse results than P2 with a lower dose [14,32].

Different types of flavonoids or polyphenols have different ranges of dose-dependent effects. Some flavonoids like galangin have a higher dosage tolerance to become pro- oxidative while other

flavonoids such as pinocembrin and chrysin have a lower tolerance on account of differing chemical structures. Interestingly, in vitro studies found that even though a higher dosage of flavonoids exerts pro-oxidative tendencies, they hold stronger anti-inflammatory properties. Propolis is known to modulate both Nrf2 and NF-κB pathways that directly contribute to their anti-inflammatory and antioxidant effects, but their crosstalk mechanism is not fully understood. It is possible that lower concentrations of phytochemicals primarily activate the Nrf2 pathway, while higher concentrations would instead inhibit the NF-κB pathway, resulting in a shift in cellular responses [14,33].

CONCLUSION

A dosage of 200 mg/kg WB/day for 14 days has proven to have a significant positive effect on the degree of interstitial fibrosis in adenine-induced CKD animal models. However, a lower dosage of 100 mg/kg WB/day for 14 days and a higher dosage of 300 mg/kg WB/day for 14 days does not exhibit a significant difference towards the control group. A higher dosage of propolis suggests eliciting dual pharmacological properties by being pro-oxidative but also exhibiting anti-inflammatory effects at the same time. Some key points remain unclear such as natural regulations concerning the interaction between Nrf2 and NF-κB pathway. It is also worth mentioning that in vivo studies in this matter are poorly perceived and would require further research.

ACKNOWLEDGEMENT

The author would like to thank all the supervisors and co-authors who contributed to this study. The author would also address their regards to Airlangga University for enabling this research.

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