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Characteristic of Streptozotocin-Nicotinamide-Induced Inflammation in A Rat Model of Diabetes-Associated Renal Injury

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Abstract

BACKGROUND: Chemical agents such as streptozotocin (STZ) and nicotinamide (NAD) are used in animal models of diabetes mellitus and their related consequences in the kidneys. Several studies have been conducted to determine the modeling, however, the results are still unclear. Moreover, diabetic nephropathy is considered to begin with an inflammatory reaction in the kidneys.

AIM: This study aims to investigate the metabolic profile STZ- and NAD-induced inflammation in the kidney.

METHODS: The male Wistar rats used were divided into control and STZ-induced diabetes. Half of the diabetes group received a single dose of nicotinamide (230 mg/kg) 15 min after STZ injection and all groups were monitored for 6 weeks. Furthermore, the profiles of creatinine, urea, and uric acid from serum and urine were observed and the kidney inflammation was tested by immunohistochemistry (IHC) with IL-6 and TNF-α parameters.

RESULTS: The result shows that the administration of a single dose of 230 mg/kg NAD in diabetic rats induced with 50 mg/kg and 65 mg/kg STZ affects body weight and kidney organ index. For 6 weeks of testing, both doses of STZ were enhanced several parameters of kidney damage in diabetic rats in blood and urine chemical parameters. Furthermore, the use of NAD to promote inflammation in STZ-induced diabetic rats gave no significant difference. However, NAD can help mice live longer and avoid problems throughout the test.

CONCLUSIONS: The use of NAD leads to inflammation in streptozotocin-induced diabetic rats. Therefore, the administration of nicotinamide is recommended since it helps the rats live longer during the experiment.

Introduction

Diabetes mellitus (DM) is a major global public health problem with increasing morbidity and death rates [1]. In 2014, it afflicted approximately 422 million individuals globally and is expected to reach 642 million by 2040 [2], [3]. The prevalence of diabetes mellitus has created several problems, particularly in kidneys disease or diabetic nephropathy (DN) [4], [5], [6]. Meanwhile, DN is a long-term consequence of both types 1 and 2 diabetes, which is beta-cell death, a complete absence of insulin [6]. According to the previous studies, DN affects more than 40% of diabetic individuals and is responsible for 45% of new occurrences of end-stage renal disease [5], [7], [8]. This has sparked a flurry of studies, including clinical trials, epidemiology, and drug development through *in vivo* study.

In pre-clinical studies, the use of streptozotocin (STZ) and nicotinamide (NAD) has been widely examined [9], [10], [11]. The results showed that STZ causes selective destruction of pancreatic beta cells, while N reduces the damage produced by STZ, which leads to partial insulin insufficiency similar to type 2 diabetes [12], [13]. In a previous study, diabetes has been modeled with different consequences, particularly in the kidneys [13], [14]. It has also been shown that nicotinamide is administered to rats to significantly prevent insulin-secreting cells against STZ, which induces pancreatic B-cell injury [15], [16]. The STZ is carried into B-cells through the glucose transporter GLUT2 to induce DNA damage, which causes the activation of the DNA repair enzyme poly (ADP-ribose) polymerase (PARP-1) [16]. However, the previous studies showed different results based on dosage, study length, receptor target, and mechanism of action. In animal studies and humans with DN, the levels of circulating inflammatory mediators and immune cell infiltration into renal tissue were higher [17]. Kidney inflammation is one of the particular targets that might signal the start of DN. Therefore, this study aims to investigate the metabolic profile of STZ- and NAD-induced inflammation in the kidney.

Methods

Materials

The chemical materials included streptozotocin and nicotinamide (Sigma Aldrich®), reagent kit glucose, creatinine, urea, and uric acid (Biosystem®).

Animals

Wistar rats were obtained from the Laboratory of Animal Life Science, Karanganyar, Central Java, Indonesia. In this study, the male Wistar rats (180–200 g) used were obtained and housed under typical laboratory settings, including a 12 h day and night light cycle. The rats were given a normal food pellet and free access to water. All animal handling procedures were approved by the ethics committee of Gadjah Mada University's Integrated Research and Testing Laboratory (No: 00034/04/LPPT/VIII/2021), which authorized this study.

STZ-Nicotinamide-induced Diabetes

The 50 Wistar rats were divided into five groups, which consist of the control and four diabetic treatment groups with 50 mg/kg and 65 mg/kg STZ induced. Meanwhile, half of the diabetes group received a single dose of nicotinamide (NAD) (230 mg/kg) 15 min after STZ injection [18], [19]. The rats with fasting glucose levels above 250 mg/dl were selected for observation (n = 10) and all groups were monitored for 6 weeks. At the end of the test, the profiles of creatinine, urea, uric acid, albumin, and total protein from urine were observed.

Bodyweight and survival rate

All animals were weighed once per 2 weeks and the number of mortality and health of the animal's test were monitored throughout the procedure time. The percent survival rate was calculated as follows: ((the rat's number of health/the number of rats in each test group) x 100) [13].

Biochemical of renal function

Meanwhile, 6 weeks after diabetes induction, the animals that survive out of all groups were euthanized. Blood samples were immediately taken from the hearts of the animals and placed in tubes. The levels of creatinine, urea, uric acid, albumin, and total protein were evaluated in serum.

Organ index observation

The rats' kidney organs were removed and weighed, meanwhile, the relative organ weight was calculated as follows: Relative organ index = (organ weight (g)/bodyweight of the animal on sacrifice day (g)).

Histopathology and immunohistochemistry (IHC) image analysis

The kidneys of the rats were carefully removed and kept in a 10% buffered formalin fixation medium for histopathology, while organ paraffin slices were produced, and stained with hematoxylin and eosin (HE) [20]. Meanwhile, the IHC testing protocol was based on the previous study by Khan *et al*. [21]. The main antibodies for the pro-inflammatory cytokines

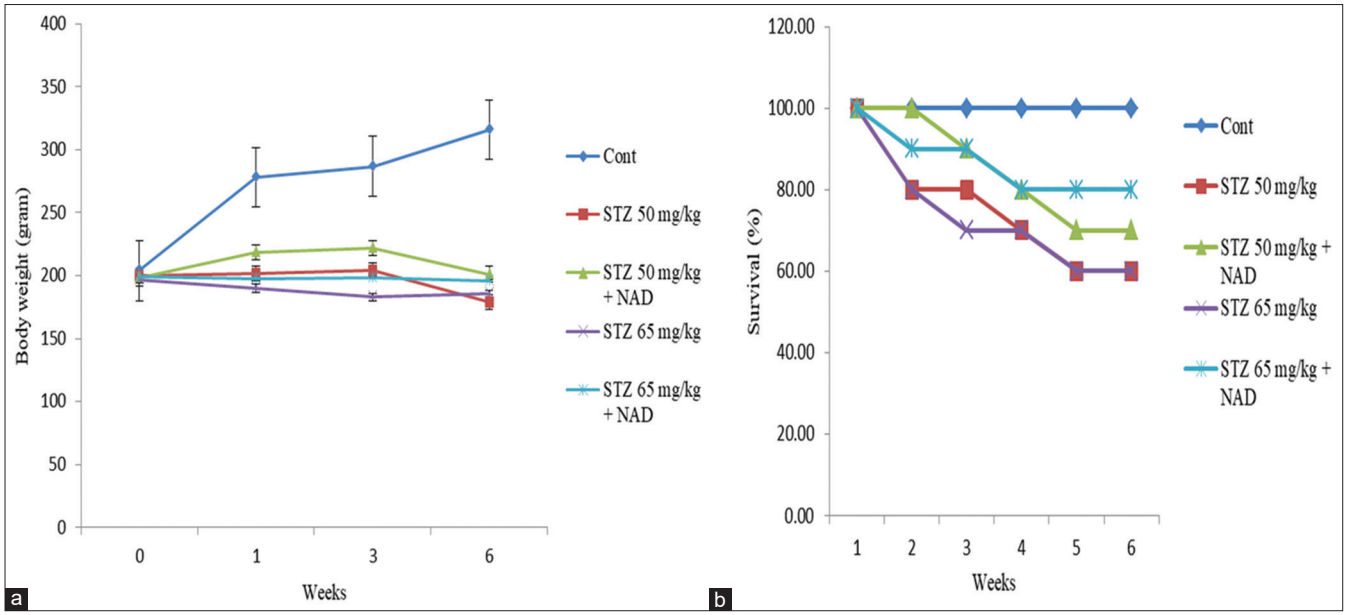

Figure 1: (a) Body weight profile (n = 6); and (b) percent survival rate of rats for 6 weeks observation. Cont (n=10), STZ 50 mg/kg (n = 6), STZ 50 mg + NAD (n = 7), STZ 65 mg/kg (n = 6), and STZ 65 mg/kg + NAD (n = 8)

Table 1: Biochemical profile of renal function in serum sample (Mean ± SD) (n = 6)

	Control	STZ 50 mg/kg	STZ 50 mg/kg + NAD	STZ 65 mg/kg	STZ 65 mg/kg + NAD
Creatinine (mg/dL)	0.83 ± 0.09	0.73 ± 0.08	0.76 ± 0.14	0.84 ± 0.13	0.99 ± 0.16
Urea (mg/dL)	68.86 ± 17.8	138.17 ± 22.08*	142.29 ± 27.8*#	74.29 ± 26.49*	114.29 ± 39.9*#
Uric acid (mg/dL)	1.77 ± 0.28	1.93 ± 0.52	1.72 ± 0.31	1.99 ± 0.83	1.77 ± 0.24

interleukine-6 (IL-6) and tumor necrosis factor-α (TNF-α) were used under observation.

Statistical analysis

The data were statistically evaluated using one-way analysis of variance (ANOVA) and recorded as mean standard deviation (SD). Duncan's *post hoc* test was also conducted to compare the results of the control group, where a significant level of $p < 0.05$ is considered statistically significant.

Results

Bodyweight and survival rate

At the end of the observation, there was an increase in bodyweight compared to starting values in the control groups. Furthermore, bodyweight loss was seen in the diabetic group throughout the procedures and was reduced by NAD (Figure 1a). In the STZ 65 mg/kg + NAD group, there was a significant difference, where the STZ 50 mg/kg + NAD dose showed a higher bodyweight profile ($p < 0.05$). The results of 6 weeks of observation showed that the administration of 230 mg/kg NAD was effective to prevent mortality rate in STZ-induced diabetic rats. The survival curves for all of the groups investigated are shown in Figure 1b.

Biochemical of renal function

Tables 1 and 2 show the biochemical parameters in diabetic rats. The test showed that only the urea levels in the serum in each group differ significantly from the control group ($p < 0.05$). The urine occurred inversely on several parameters including creatinine, urea, and uric acid, which showed a significantly decreased amount compared to the control group ($p < 0.05$). Furthermore, serum urea levels in diabetic rats with 50 mg/kg and 60 mg/kg STZ-induced decreased slightly higher when combined with NAD. In most of the other serum biochemical parameters, there was no increase, therefore, it was considered significant for the control group.

Organ index

Based on the observation results as shown in Figure 2, the kidney organ index of diabetic rats correlated with the bodyweight. In control, rats showed the smallest index, while the diabetic group showed a decrease in bodyweight and an increase in organ index values. The STZ- and NAD-induced diabetic rats showed lower index values compared to the group without treatment.

Histopathology and IHC image

Figure 3 shows a graph of the expression of inflammatory mediators (IL-6 and TNF-α) in rat kidney organs after 6 weeks of observation. When compared to the control group, the graphs show the presence of inflammation in the renal cortex, which is characterized by a considerably elevated production of IL-6 and TNF-α ($p < 0.05$). Meanwhile, the administration of NAD in STZ-induced diabetic rats at doses of 50 and 65 mg/kg did not show a significant difference other than TNF mediators at a dose of 50 mg/kg. The histological profile (Figure 4) showed a linear condition, where the medulla and renal cortex of the control group appeared normal. Tubular and glomerular cells of the control group appeared normal with minimal inflammation (Figure 4.1a-b). In diabetic rats induced by STZ 50 mg/kg, tubular cells appeared swollen and necrotic with moderate lymphocytes and macrophages. Glomeruli with moderate inflammatory cells shown in Figure 4 (2a.b). In the medulla and renal cortex of 50 mg/kg STZ-induced and 230 mg/kg, NAD diabetic rats showed mild inflammatory cell infiltration (Figure 4.3a-b). Kidney tubular cells of diabetic rats with 60 mg/kg STZ-induced appeared swollen and partially degenerative, with few lymphocytes and macrophages as shown by the glomerulus with moderate inflammatory cells (Figure 4.4a-b). Meanwhile, in diabetic rats with 60 mg/kg STZ-induced and the addition of 230 mg/kg NAD, the medulla and cortex were infested with mild-moderate inflammatory cells (Figure 4.5a-b).

Table 2: Biochemical profile of renal function in urine sample (Mean ± SD) (n = 6)

	Control	STZ 50 mg/kg	STZ 50 mg/kg + NAD	STZ 65 mg/kg	STZ 65 mg/kg + NAD
Creatinine (mg/dL)	3.7 ±	0.49 ± 0.17*	0.55 ± 0.27*	0.60 ± 0.17*	0.44 ± 0.2*
Urea (mg/dL)	164.69 ±	57.27 ± 22.4*	56.57 ± 8.46*	26.09 ± 13.9*	18.48 ± 8.67*#
Uric acid (mg/dL)	3.20 ±	1.95 ± 0.34*	1.89 ± 0.26*	1.92 ± 0.3*	1.85 ± 0.21*

*Indicates a significant difference between diabetes dose and control groups ($p<0.05$); #Shows a significant difference between the administration of NAD to the group of rats that were not given a dosage of 50 mg/kg STZ and 65 mg/kg STZ.

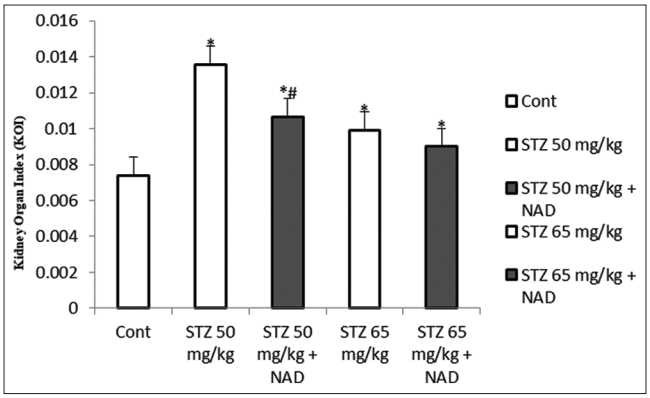

*Figure 2: Kidney organ index in rats for 6 weeks observation (n=6). Description: *Indicates a significant difference between diabetes dose and control groups (p < 0.05); #Shows a significant difference between the administration of NAD to the group of rats that were not given a dosage of 50 mg/kg STZ and 65 mg/kg STZ*

Discussion

The results showed that a single 230 mg/kg NAD dose administered after 50 mg/kg and 65 mg/kg STZ-induced diabetes can reduce some of the blood and urine biochemical abnormalities in the model. Meanwhile, the main novelty of this study is the impossibility of constantly increasing the dose of STZ used in inducing diabetes in rats to produce linear kidney complications. However, the effect of NAD treatment in STZ-induced rats increased much blood and urine biochemicals. After 6 weeks of observation, there were no significant changes in the occurrence of inflammation in the kidney organs of rats at doses of 50 mg/kg and 65 mg/kg with the addition of NAD or not. Meanwhile, NAD has been shown to decrease mouse mortality throughout the test. According to Cruz *et al*. (2021), the death inhibitory effect was related to maintaining baroreflex and parasympathetic sensitivity regulation [13].

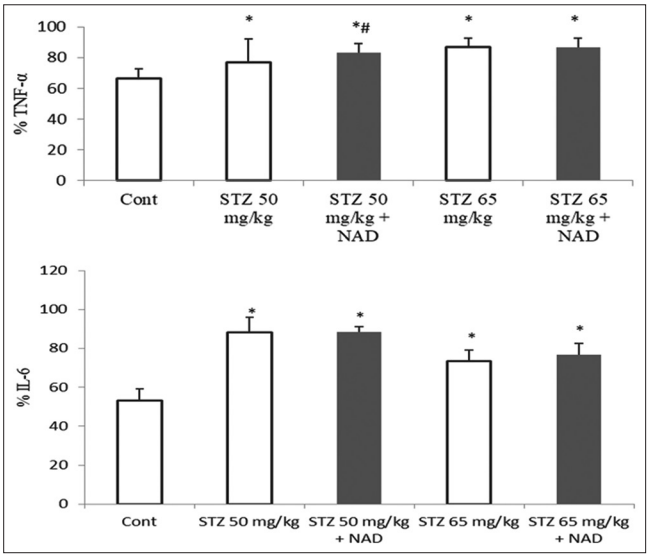

*Figure 3: Kidney inflammation by immunohistochemistry imaging. Description: *Indicates a significant difference between diabetes dose and control groups (p < 0.05); #Shows a significant difference between the administration of NAD to the group of rats that were not given a dosage of 50 mg/kg STZ and 65 mg/kg STZ*

Streptozotocin is a highly cytotoxic agent to rat pancreatic beta-cells, which induces DNA damage in insulin-secreting cells, causing DNA repair processes, mitochondrial malfunction, and ATP depletion [21], [22]. Therefore, rats exhibit severe diabetic signs such as elevated glycemia and bodyweight loss [23], [24]. This is in line with the test results, which showed that STZ doses of 50 mg/kg and 65 mg/kg produce significant weight reduction in rats model diabetic (Figure 1a). Furthermore, problems such as kidney injury occur in diabetic animal models with uncontrolled blood sugar levels [25], [26]. Several studies showed that STZ-induced diabetic rats with blood glucose levels >250 mg/dl showed a nephropathy effect [4], [27], [28]. Meanwhile, in this study, the results indicated that the induction of diabetes with STZ and STZ+NAD for 6 weeks has not shown the occurrence of diabetic nephropathy symptoms on most parameters. However, in some parameters such as serum urea levels, there was a significant increase (Table 1 and 2). The common parameters to indicate kidney health include chemical urine levels such as creatinine, urea, and uric acid [29], [30]. Meanwhile, kidney injury can be detected by a change in chemical levels that are above normal [28].

In this study, observations were also made on inflammatory biomarkers such as IL-6 and TNF-α. Although DN is generally assumed to be a non-immune illness, accumulated data showed that immunological and inflammatory processes play an important role in its progression [18], [31], [32]. Inflammatory mediators are mostly generated by peripheral blood mononuclear cells, however, they are also produced by renal cells. The involvement of abnormal cytokine and chemokine release in the main immunopathological processes of DN has been shown [32]. Therefore, inflammatory mediators such as TNF-α, IL-6, and other markers engaged were identified as possible indicators of progressive DN [33]. This study also showed that the expression of cytokine IL-6 and TNF-alpha increased in kidney cells in all diabetic groups (Figure 3). The addition of NAD in STZ-induced diabetic rats at doses of 50 mg/kg and 65 mg/kg did not show a significant difference in the expression of inflammation. Furthermore, the low-grade inflammation correlated with blood and urine chemistry values was a risk factor for the development of DN. TNF-α is an important inflammatory mediator in tissue damage, which is expressed, synthesized, and released by infiltrating macrophages in the kidney organs, specifically endothelial cells, tubular epithelium, glomerulus, and mesangial [34], [35], [36]. In addition, TNF-α was identified as a key factor in the pathophysiology of kidney damage, which increases inflammation, apoptosis, the development of extracellular matrix, and decreases glomerular blood flow, also

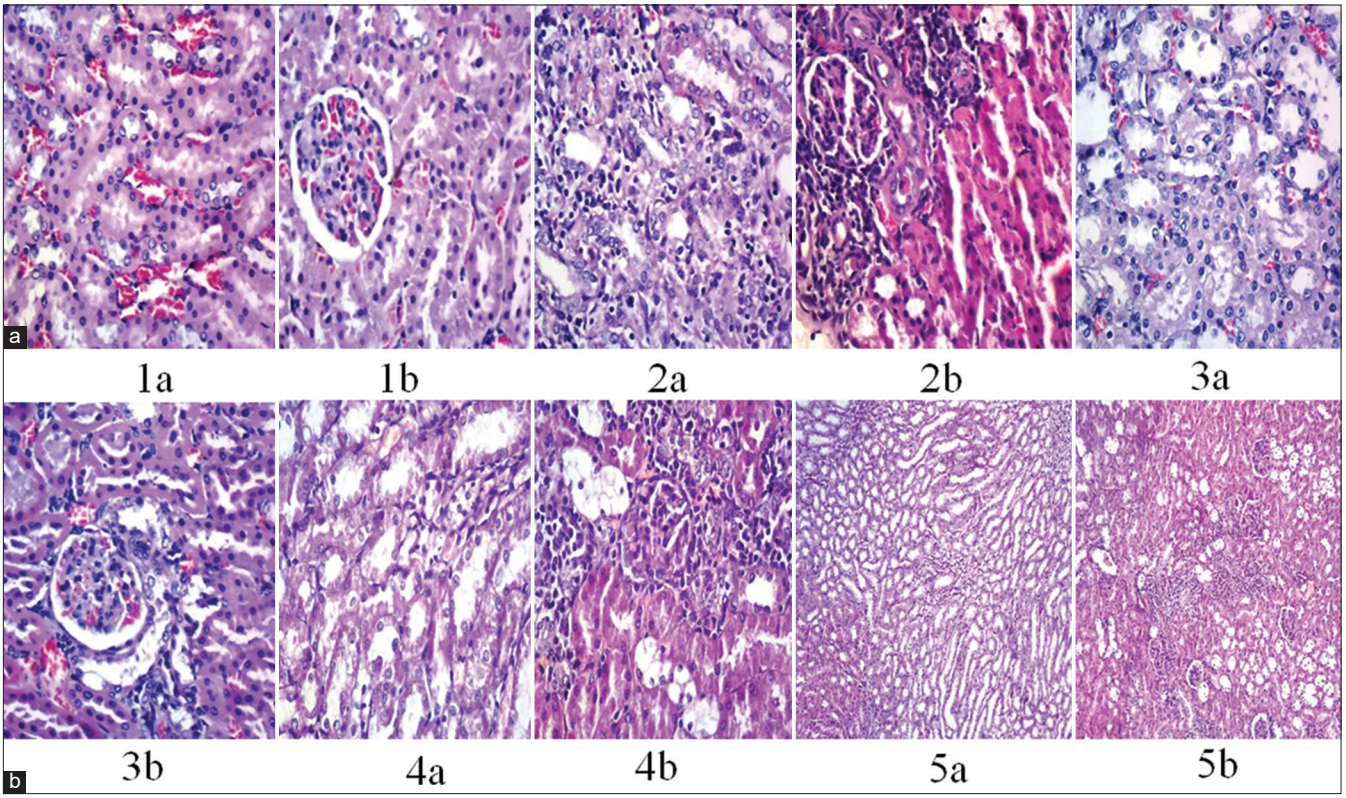

Figure 4: Histopathological changes in kidney islet of normal and treated rats (hematoxylin and eosin; 10×). 1. Cont; 2. STZ 50 mg/kg; 3. STZ 50 mg/kg+NAD; 4. STZ 65 mg/kg; and 5. STZ 65 mg/kg+NAD; (a) medulla and (b) cortex

weakens the glomerular permeability barrier [37], [38]. Meanwhile, several studies stated that IL-6 was more in patients with DN than without DN [39], [40].

This study has several limitations, which include the use of many methods to induce diabetes in rats. Furthermore, two doses of STZ, namely, 50 mg/kg and 65 mg/kg with or without the addition of NAD, were presented. High doses of >65 mg/kg were also used, but it did not take time for the mice to die. This showed that it is not possible to present more accurate data at these high doses. The additional dose of NAD of 230 mg/kg is also based on the previous studies, therefore, it does not present a new dose [4]. In the blood and urine chemistry values, only parameters specific to the kidney organs, namely, creatinine, urea, and uric acid, were shown because they are general markers and are often reviewed in many studies. Since day 7 of STZ administration, high blood sugar has been reported in many studies to remain high until the end of the test [28]. In addition, the urine and blood ketone bodies of the subjects were not measured. Therefore, further studies dealing with long-term effects are recommended.

Conclusions

Streptozotocin-induced diabetic rats using NAD lead to kidney inflammation similar to diabetic conditions. Based on the results, most of the parameters of kidney injury in serum samples did not show significant differences after the addition of NAD in rats STZ induced at moderate to high doses. However, in urine samples, there was an increase in the same parameters at all doses. Therefore, nicotinamide administration is recommended since it helps the rats live longer during the experiment.

Institutional review board statement

The study was approved by the Ethics Committee of Gadjah Mada University's Integrated Research and Testing Laboratory (No: 00034/04/LPPT/VIII/2021).

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