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Regenerative alveolar bone in dental sockets of diabetic wistar rats post tooth extraction

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ABSTRACT

Background: The bone healing of the alveolar socket post-dental extraction is critical in dental services. Delayed healing is a common problem in diabetes mellitus (DM) patients. It is due to the impaired angiogenic response and microvascular complications. Okra fruit extract can control diabetes mellitus (DM) in several ways, delaying glucose absorption and regenerating pancreatic cells. It can impact an increase in insulin secretion and glycogenesis to control hyperglycemia in DM. This study analyzed the regenerative alveolar bone through the expression of TGF-β2 in the tooth socket of Wistar rats with diabetes mellitus post-extraction after administration of okra fruit extract.

Patients and methods: Extraction of the rat mandibular left incisor was performed using a pair of modified forceps. The tooth sockets were then rinsed using a saline solution. Two groups (control & treatment) of four rats were sacrificed on days 3, 5, and 7. The socket tissues from the rats were then immunohistochemically analyzed

Results: The average level of TGF-β1 expression in the treatment (T) groups was higher compared to the control (C) group: day 3 (10.50±2.67 versus 4.83±1.17), day 5 (12.33±1.63 versus 5.17±2.32), and day 7 (13.00±2.83 versus 4.50±1.05), with P = 0.000

Conclusion: The administration of okra fruit extract can increase regenerative alveolar bone healing through increased expression of TGF-β2 in the dental socket after tooth extraction of diabetic Wistar rats.

Keywords: Diabetes mellitus, okra fruit, TGF-β2, wound healing, bone regenerative.
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INTRODUCTION

Bone tissue regeneration is an important problem in medicine and dentistry, one of which is abnormalities due to infectious diseases. One of the goals of treating bone defects is to restore the normal morphology and function of the damaged structures. The process of bone formation goes through two phases, namely endochondral ossification and intramembrane ossification.[1] An excellent balance between osteoblasts and osteoclasts are the two primary bone cells involved in the process of bone remodeling. Osteoblasts are responsible for bone formation, while osteoclasts are bone-absorbing cells.[2]

Wound healing is a dynamic process that completely restores anatomic integrity with other functions without spreading infection. Acute wounds generally heal quickly without causing problems if there are no disturbances in physiological functions such as poor blood circulation, obesity, diseases such as diabetes, and stress. Chronic wounds are tissue wounds that do not heal in an organized series of stages and take more than 12 weeks to heal.[3,4] In general, the healing process begins with hemostasis due to blood loss and microbial invasion of the wound area. This phase is followed rapidly and overlaps with the inflammatory phase, which initially begins with the infiltration of proinflammatory neutrophil cells, followed by macrophages, the release of growth factors and cytokines, and other cells. The proliferative phase overlaps with the inflammatory phase. It includes new tissue and blood vessels (angiogenesis), and matrix construction is initiated to fill the injured area. The final remodeling phase then increases the tensile strength of the extracellular matrix and reduces blood supply to the damaged area.[5]

Diabetes mellitus (DM) can disrupt various wound-healing processes. It includes hemostasis, inflammation, proliferation, and remodeling phases. This problem will impact long-term adverse effects on quality of life, morbidity, and mortality characterized by chronic wounds that cause impaired healing due to delayed or uncoordinated healing. DM shows a persistent inflammatory phase due to obstacles in forming granulation tissue and reduced wound tensile strength caused by damage to blood vessels that cause ischemia.[6]

Diabetic disease can delay the healing process leading to non-healing wounds. Some various complications are identified, such as functional impairment and infections. The common infections in diabetic patients are cellulitis, abscesses, osteomyelitis, gangrene, and septicemia.

The wound-healing process requires collaboration between inflammatory cells and biochemical mediators stimulated by various factors. Changes in cellular and biochemical factors and their activities have been implicated in the failure of wound healing in diabetics. The cells involved in wound healing are neutrophils, monocytes, macrophages, keratinocytes, fibroblasts, T cells, B cells, mast cells, and endothelial cells. These cells actively produce and regulate various cytokines and growth factors. Monocytes, which then turn into macrophages, produce proinflammatory cytokines IL-1β, TNF-, IL-6, and VEGF5 IGF-1. TGF-β is the main factor in both diabetic and non-diabetic conditions. Neutrophils and T and B cells are also significant producers of TNF-, IL-10, and other cells, keratinocytes, fibroblasts, mast cells, and endothelial cells. These cells also contribute to the production of VEGF, IGF-1, and TGF-β.[7]

Studies reveal that in diabetes, a complex mechanism involving the molecular level is responsible for delayed wound healing. Activities include sustained production of proinflammatory cytokines, impaired angiogenic response, and microvascular complications.[6] It also impaired macrophage and neutrophil function,[8] migration and proliferation of keratinocytes, fibroblasts, and impaired factor production Cure-related disorders such as impaired growth factor production have been reported in animal models of diabetes.[9] Macrophages are essential contributors to healing. Hyperglycemia and oxidative stress alter the epigenetic code, resulting in macrophage polarization and modulation changes. Dysregulated macrophage polarization is one of the main reasons for delayed wound healing.[10,11]

Okra fruit contains chemicals such as saponins, tannins, alkaloids, and flavonoids. In addition, okra has quercetin which functions as an antioxidant and antitumor. Its antioxidant properties inhibit endothelial cell migration.[12] Saponins are another active substance in okra, which have antibacterial activity and stimulate angiogenesis. Furthermore, it was shown that flavonoids, mediators of type III collagen synthesis, act as anti-inflammatory agents, modulate oxidative burst in neutrophils, and act as phospholipase inhibitors.[13] Flavonoids also reduce reactive oxygen species (ROS). Therefore, it can potentiate wound healing.[14]

This study aims to analyze the expression of growth factor TGF-β1 in post-extraction tooth sockets in diabetic Wistar rats.

MATERIAL AND METHODS

Research Design and Animal Model

This study is a laboratory-based of experimental analytic study. Wistar rats used as samples in this study were obtained from the Experimental Animal Unit of the Biochemical Laboratory of the Faculty of Medicine, Universitas Airlangga. Research Ethical Committee Faculty of Dental Medicine Universitas Airlangga approved the study (No. 561/HRECC.FODM/X/2021).

Collection, adjustment, maintenance, and treatment were carried out in the Experimental Animal Unit of the Biochemical Laboratory of the Faculty of Medicine, Universitas Airlangga. Okra fruit was extracted in Materia Medika Batu, Malang, East Java. Histological preparations were carried out at the Anatomy Pathology Laboratory of the Faculty of Medicine, Universitas Airlangga.

Immunohistochemistry staining was used to examine the TGF-β2 expressed cell. The examination was conducted at the Laboratory of Biochemistry, Faculty of Medicine, Universitas Brawijaya, Malang.

Okra fruit extract preparation

Fresh okra fruit was dried in a drying oven until a constant weight was reached. The dried fruit was then ground into powder. A total of 2 grams of powder was extracted with 20 ml of 70% ethanol in a ratio of 1:10 (w/v) during the maceration period (24 hours) at room temperature. The solvent and soaked powder mixture was filtered through filter paper and then concentrated to 1 ml with a rotary evaporator and diluted with 5% dimethyl sulfoxide (DMSO) at a ratio of 1:1 (v/v). The results were stored at a temperature of minus 20 OC until further use.[15,16]

Research Procedure

In this study, 24 male Wistar rats aged 2-3 months with a weight of 150-200 grams were adapted in the same cage at 25 ± 2 □C. The 24 Wistar rats were divided into two groups (control group and treatment group). The rats were supplied with standard pellet food and distilled water ad libitium for seven days and 4 hours before being induced with streptozotocin (STZ) (Nacalai Tesque Inc., Japan). The 2% STZ solution was dissolved with 0.1 mol/L citrate buffer solution pH 4.4 at a 45 mg/kg dose and converted to a dose of 6.75 mg/150gr. The solution was then administered to the Wistar rats through intraperitoneal induction. Blood glucose levels were measured on day three after STZ induction by taking a blood sample from the lateral veins in the rats' tails. Measurements were performed using a glucometer (Accu Chek® Instant). The Wistar rats were diagnosed with DM if the blood glucose levels were ≥ 200 mg/dl after the STZ induction (Qinna and Badwan, 2015).[17]

Wistar rats with DM were then anesthetized through peritoneal injection using 0.1 ml of ketamine per rat. After the injection, a resting period of 1-1.5 hours was given, after which extractions of the rats' mandibular left incisors were performed using a pair of modified forceps and an elevator. The tooth sockets were then irrigated with saline solution.[18]

In the control group, the animals did not receive the administration of okra fruit extract.

Instead, they were only supplied with distilled water prior to the observation. KO1 was observed on the third day, KO2 was observed on the 5th day, and KO3 was observed on the seventh day. In the treatment group, the rats were given oral administration of okra fruit extract after the tooth extraction with a dose of 250 mg/kg, which was converted to a dose of 37.5 mg/150 gr once a day. PO1 was observed on the third day, PO2 on the fifth day, and PO3 on the seventh day.

Wistar rats were sacrificed on the 3rd, fifth and seventh day using a lethal injection of intraperitoneal ketamine (no less than four times the anesthetic dose or about 0.4 ml/kg). The mandibular of each

rat was taken from the temporomandibular joint. After this, the Wistar rats were buried according to the ethical treatments of experimental animals. The mandibles in the incisor area were cut vertically and treated with paraffin.[19] The immunohistochemical examination using the method used by Luthfi et al.[20] We count the expressed cell in a Light microscope with 400x magnification. The expressed cell number was evaluated on 4 microscopic fields, and the average of TGF-β2 expressed cells per microscopic field was analyzed statistically.

Statistical Analysis

Statistical analysis was performed using SPSS (IBM SPSS Statistics for Windows, Version 24.0: IBM Corp., USA). The Shapiro-Wilk test was used to find out normally distributed data. After the distribution test, Levene's homogeneity test was then performed. The data that showed normal distribution was homogeneous, and then analysis was continued with the One Way ANOVA test and multiple comparison tests using Tukey HSD.

RESULTS

The study ended in 7 days of the experiment. There was any Wistar rat that died during the study. The data of the TGF-β2 expressed cell we collected and tabulated and statistical analyses. Tables 1 and 2 show that all the data are typically distributed based on the Shapiro-Wilk test (p = 0.124) and homogeneous based on the Levene test (p = 0.533). There is no impact of the interaction between time and treatment against TGF-β2 cell expression (Table 4; p>0.05). However, shows a significant difference between the control versus treatment groups (Figure 1; Table 5; p=0.000).

The study has been conducted, control versus treatment, to evaluate the effect of okra fruit extract in the healing process of the dental socket post extraction. The blood glucose level of all rats was above 200mg/dl after the induction.

The TGF-β2 expression appears as gradients of yellow to brown stains (red arrow). Figure 2 shows that on the third day, the PO1 group showed an increasing number of TGF-β2 expressions compared to the KO1 group. On the fifth day, PO2 showed an increasing number of TGF-β2 expressions compared to the KO2 group. On the seventh day, PO3 showed an increasing number of TGF- β2 expressions compared to the KO3 group.

Table 1. Shapiro test for normality of TGF-β2 expression in control and treatment groups

	Kolmogorov-Smirnov			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	Df	Sig.
Residual for **TGF-β2**	.108	36	.200	.952	36	.124

Table 2. Levene test Homogeneity of the data of TGF-β2 expression cell in control and treatment group.

Group	N	Mean ± Standard Deviation	Sig.
KO1	6	4.8333 ± 1.16905	0.533
KO2	6	5.1667 ± 2.31661	
KO3	6	4.5000 ± 1.04881	
PO1	6	10.5000± 2.66458	
PO2	6	12.333 ± 1.63299	
PO3	6	13.000 ± 2.82843	

Notes::*: KO=Control group; PO=Treatment group; 1,2,3 are day 3, 5 and 7; N = number of Wistar rats per group.

Table 3. Descriptive data of the mean and standard deviation of the treatment and control groups based on days of evaluation

Days Evaluation	N	Group	Mean ± SD
3	6	KO1	4.8333±1.16905
	6	PO1	10.5000±2.66458
5	6	KO2	5.1667±2.31661
	6	PO2	12.3333±1.63299
7	6	KO3	4.5000 ±1.04881
	6	PO3	13.0000±2.82843

Note: N=the number of rat in each group; KO = Control; PO = Treatment

Table 4. Two way ANOVA test between control and treatment groups and their interactions

Variables	P value	Eta squared
Time	0.346	.068
Group	0.000	.780
Time * Group	0.259	.086

Table 5. Comparative test results of TGF-β2 based on treatment

Group	Mean ± SD	Sig.
Treatment	4,83 ± 1,54	0.000
Control	11,94 ± 2.53	

DISCUSSION

Routine wound healing is a well-coordinated and synchronized process by factors. It includes growth factors, matrix metalloproteinases (MMP), cytokines, neutrophil cells, proinflammatory

macrophages, keratinocytes, fibroblasts, and endothelial cells. Growth factors are biologically active polypeptides that are involved in all phases of the healing process.[21] Growth factors occur in the early inflammatory and granulation phases, namely tissue formation. Wound healing disorders are caused by defects in the type and growth factors. This healing disorder is due to changes in expression, decreased production, release, trapping, and excessive degradation.[22]

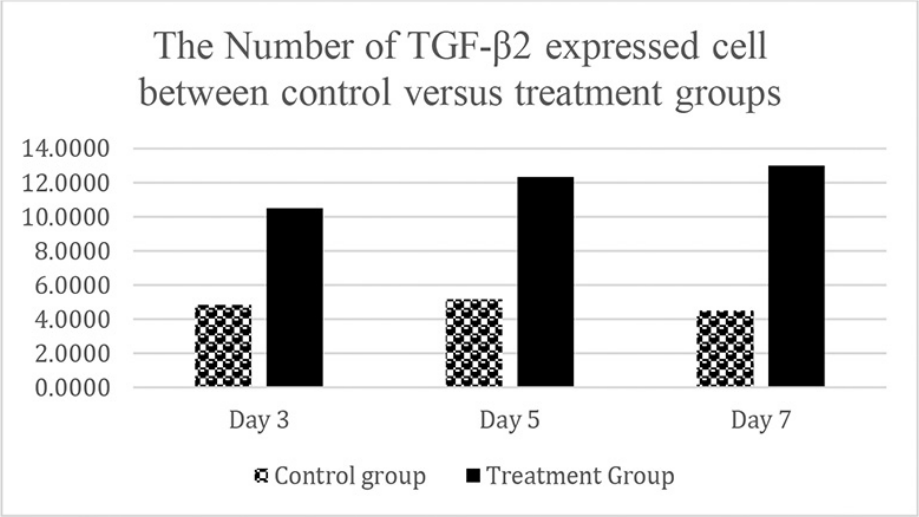

Figure 1. Graph the average of TGF-β2 expression cells on days 3, 5, and 7 in the socket tissue of Wistar rats in the control group and the treatment group

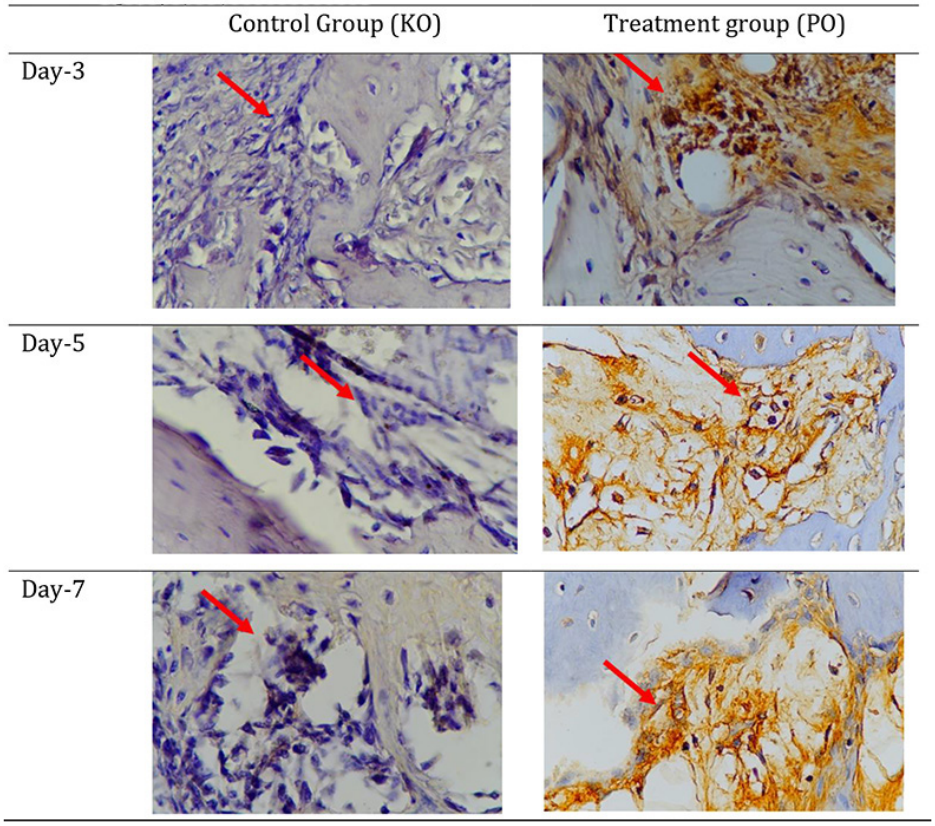

Figure 2. Expression of TGF-β2 days 3,5 and 7 in the socket tissue of Wistar rats with Diabetes Mellitus on immunohistochemical examination under a 400x magnification microscope in the control group (KO) and the treatment group (PO).

The balance between matrix formation and matrix degradation characterizes ECM synthesis with optimal healing. Factors that regulate ECM formation, such as VEGF, IGF-I, IGF-II, TGF-β, KGF24, PDGF25, EGF26, and FGF27 stated that TNF- and IL-6 markedly decreased in diabetic patients.[23] Growth factors are essential in initiating and maintaining the different phases of wound healing. Any changes i.e., down-regulation of growth factor receptors and rapid degradation of growth factor, cause delayed wound healing in diabetics.

The flavonoid is an antioxidant in okra fruit extract (Abelmoschus esculentus). It shows a high reactive hydroxyl group that can react with the reactive component of free radicals so that the formation of ROS can be stable and controlled, characterized by a balance between oxidants and antioxidants.[24] Flavonoids also act as anti-inflammatory agents through immunomodulatory mechanisms. Flavonoids can increase Th-1 and Th-2 production and IFN production, which can induce the formation of M1 and M2. M1 formed can play a role in phagocyte apoptosis from neutrophils. An induced shift from phenotype M1 macrophages to M2 results in growth factors such as VEGF. Anti-inflammatory mechanisms in flavonoids can also reduce the activity of cyclooxygenase, which causes reduced prostaglandin synthesis, so the inflammation process increases.[25]

The low expression in the control group compared to the treatment group is due to the diabetic state after injection of hyperglycemia, which causes the formation of Advanced Glycation End Products (AGEs). If AGEs bound to the Receptor for Advanced Glycation End Products (RAGE) will increase the activation of Mitogen-Activated Protein Kinases (MAPKs), Phosphatidylinositol-3 Kinases (P13-K), and Nicotinamide Adenine Dinucleotide Phosphate Oxidase (NOX) which are the primary sources of the formation of ROS (Luevano-Contreras et al., 2013; Sanches et al., 2018). This Mechanism can lead to activation (NF-kβ which transcribes proinflammatory cytokines such as TNF-α, IL-6, and IL-

1β. In diabetic Wistar rats with chronic lesions, the ability of macrophages to neutralize neutrophils decreases so that they cannot induce shifting from M1 to M2. The increase of proinflammatory caused by the activation of NF-kβ and dysregulation of shifting M1 to M2 can cause Wistar diabetic mice to experience a persistent inflammatory phase so that it becomes chronic inflammation.[26]

Okra fruit contains other substances, such as quercetin, which besides acting as an antioxidant, also has antitumor properties. Antitumor properties of quercetin relate to its ability to prevent tumor vascularization by inhibiting the growth and migration of endothelial cells.[27] Okra fruit extract at 30% can promote angiogenesis in post-extracted tooth sockets of Wistar rats.[20]

The proliferation phase comprises re-epithelization, angiogenesis, granulated tissue formation, and collagen deposition, starting on day four until two weeks after injury. The proliferation phase denotes the earlier-formed matrices formation during the hemostasis. It will be replaced by granulated tissue consisting of fibroblasts, granulocyte, macrophage, and newly-formed capillaries. The migration and proliferation of fibroblast cells constitute a response toward the growth factors, namely platelet derivate growth factor (PDGF), tumor growth factor-β (TGF-β), b fibroblast growth factor (bFGF), which are secreted by platelets and macrophages. Fibroblast will proliferate and secrete proteinase. Such as matrix metalloproteinase (MMP) to degrade the surrounding matrices and replace them with collagen and extracellular matrices (ECM).[28,29]

In this study, on days 3 and 5, and 7, we observed an increase of TGF-β2 due to the flavonoids in okra. It can reduce the release of prostaglandins and proinflammatory mediators by inhibiting the cyclooxygenase enzyme.[30] In addition, flavonoids can also reduce oxidative stress by regulating the activity of NF-kB so that the increase in proinflammatory cytokines can be reduced. The decrease causes inducible Nitric Oxide Synthase (iNOS) activity to be suppressed to accelerate the wound-healing process.[28]

A previous study by Pang et al. found that low-dose flavonoids alone could stimulate the expression of TGF-β growth factor, which increased TGF-β levels until the seventh day and in the wound healing process is a stimulator of fibroblast cells.[31] Giving okra 30% fruit extract gel in the tooth socket after extraction can increase fibroblast cell proliferation.[20]

The world has seen a rapid increase in the incidence of diabetes generally, and type 2 diabetes has become the most common metabolic disease globally. The increased diabetic state has been partly due to a growth in obesity. It is especially also due to the excess visceral adiposity. In addition, the associated "metabolic syndrome" encompasses insulin resistance, hyperglycemia, dyslipidemia, and hypertension.[32]

Inflammation occurs in response to various pathological stimuli and tissue injury. Chronic inflammation and immune system activation may be mainly responsible for obesity-related metabolic diseases such as type 2 diabetes.[32,33] Critical characteristics of type 2 diabetes include insulin secretion defects and insulin resistance in the liver, adipose tissue, and skeletal muscles. Diabetes and associated complications result from the inflammatory processes.[34]

The antidiabetic qualities of quercetin involve the stimulation of glucose uptake through a MAPK insulin-dependent mechanism. Stimulating the Mechanism in skeletal muscles has resulted in glucose transporter 4 (GLUT4) translocation. This role for MAPK is distinct from its role in the liver, where it reduces sugar production mainly through the downregulation of vital gluconeogenesis enzymes (Haddad et al., 2015).[35]

Studies have been conducted on the effects of quercetin in animals with type 2 diabetes.[36] Those who received quercetin showed lower glucose plasma levels than the control group and did not experience an increase or decrease in insulin measured by the homeostasis model. Animals that received a 0.08% portion of quercetin showed various other improvements. It includes an increase in plasma adiponectin HDL-cholesterol, decreases in plasma total cholesterol and triacylglycerols, and increases in specific liver enzyme activities important in detoxification. Quercetin is pivotal in improving renal function in diabetic nephropathic rats by blocking the overexpression of connective tissue growth factor (CTGF) and transforming growth factor-β1 (TGF-β1). End-stage renal disease is closely associated with diabetic nephropathy. Studies show that TGF-β1 and CTGF have an essential impact on the DN pathophysiological systems. Studies examined the impact of quercetin on TGF-β1, and CTGF renal functions in streptozotocin (STZ) induced diabetic Sprague-Dawley rats.[37] Results showed that rats treated with quercetin reduced their kidney and body weight ratio. The expressions of CTGF and TGF-β1 are higher in the renal tissues. For those that received quercetin, the overexpression was decreased.

Finally, quercetin has been shown to produce an effective in vitro block against lens aldose reductase and prevent polyol accumulation.[38] For humans, quercetin has been shown to help decrease the seriousness of numbness, jolting pain, and irritation for patients with type 2 diabetes neuropathy. It has further shown that active quercetin treatment can improve various quality-of-life matrices.[39]

CONCLUSION

Administration of okra fruit extract increased the expression of TGF-β2, which indirectly increased alveolar socket bone healing in the context of regenerative dentistry.

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DISCLOSURE

There is not any conflict of interest.

AUTHOR CONTRIBUTION

All author contributed in the research.

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