Histomorphometric and immunohistochemical evaluation of the frontal cerebral cortex in diabetic rats after treatment with melatonin

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ABSTRACT. Baptista M.G.P., Ferreira C.G.M., Albuquerque Y.M.L., D’assunção C.G., Alves R.C., Wanderley-Teixeira V. & Teixeira A.A.C. 2020. Histomorphometric and immunohistochemical evaluation of the frontal cerebral cortex in diabetic rats after treatment with melatonin. Pesquisa Veterinária Brasileira 40(12):1077-1087. Departamento de Morfologia e Fisiologia Animal, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: marinaabaptistaa@gmail.com

The central nervous system is vulnerable to complications caused by diabetes. These complications lead to increased oxidative stress in the brain, resulting in damage to the cerebral cortex, among other regions. Insulin and hypoglycemic agents are still the most widely used treatments. However, current research with an experimental model of diabetes suggests the use of antioxidants, such as melatonin. Thus, we tested the hypothesis that exogenous melatonin may decrease or prevent the effects of diabetes in the frontal cortex of the rat brain. Fifty albino rats were allocated into five groups: GC = rats without diabetes induction, GD = diabetic rats induced by streptozotocin, GDM = streptozotocin-induced and melatonin-treated diabetic rats, GDI = diabetic rats induced by streptozotocin and treated with insulin, GDMI = diabetic rats induced by streptozotocin and treated with melatonin and insulin simultaneously. Diabetes was induced by intraperitoneal administration of streptozotocin (60mg/kg). Insulin (5U/day) was administered subcutaneously and melatonin (10mg/kg) by drinking water; both treatments last days after. We analyzed animals’ weight, the cytokines IL-6 and TNF-α, apoptosis, glycogen, and did morphometry and histopathology of the frontal cortex were analyzed. The results showed that the cerebral cortex of the diabetic animals presented axonal degeneration, reduced number of neurons in the cortex, reduced glycogen, increased IL-6 and TNF-α expression, high apoptotic index, and reduced animal weight and the brain. Treatment with melatonin associated or not with insulin prevented such effects. Thus, we conclude that melatonin associated with insulin may be an alternative for avoiding the impact of diabetes in the brain’s frontal cortex.

INDEX TERMS: Frontal cerebral cortex, diabetes, brain, melatonin, cytokines, apoptosis, morphometry, rats, Brazil.
tratados com melatonina, GDI = ratos induzidos ao diabetes pela estreptozotocina e tratados com insulina, GDMI = ratos induzidos ao diabetes pela estreptozotocina e tratados com melatonina e insulina simultaneamente. O diabetes foi induzido pela administração intrapertoneal de estreptozotocina (60mg/kg). A insulina (5U/dia) foi administrada por via subcutânea e a melatonina (10mg/kg) pela água de beber. Ambos tratamentos foram realizados durante 30 dias após a indução. Foram analisados o peso dos animais, do cérebro, as citocinas IL-6 e TNF-α, apoptose, glicogênio, além da morfometria e histopatologia do córtex frontal. Os resultados mostraram que o córtex cerebral dos animais diabéticos apresentou degeneração axonal, redução do número de neurônios no córtex, redução do glicogênio, aumento da expressão do IL-6 e TNF-α, elevação do índice apoptótico, além da redução do peso dos animais e do cérebro. O tratamento com melatonina associada ou não a insulina preveiu tais efeitos. Assim, concluimos que a melatonina associada ou não a insulina pode ser uma alternativa na prevenção dos efeitos do diabetes no córtex frontal do cérebro.

TERMOS DE INDEXAÇÃO: Córtex cerebral frontal, diabetes, cérebro, melatonina, citocinas, apoptose, morfometria, ratos.

INTRODUCTION

For several years, interest in the effects of diabetes on the brain has increased significantly. The central nervous system is vulnerable to complications caused by diabetes caused by chronic hyperglycemia (Van–Harten et al. 2006, Francis et al. 2008, Koldi et al. 2008, You et al. 2009, Yang et al. 2011, Huang et al. 2012, Yoon et al. 2017). Several studies have shown that vascular and metabolic changes associated with chronic hyperglycemia can cause brain damage, cerebral atrophy, structural and electrophysiological changes, and impairment of cognitive function (Van-Elderen et al. 2010, Franc et al. 2011, Louzada & Vargas 2015). Although several studies report adverse effects of diabetes mellitus on the hippocampus, hypothalamus, and cerebellum (Jackson-Guilford et al. 2000, Piotrowski et al. 2001, Beaufu et al. 2006, Khaskar et al. 2010, Ahmadpour & Haghir 2011), research has shown that diabetic patients have lesions in the white matter of the frontal cortex of the brain (Hsu et al. 2012).

Diabetes stimulates neuron damage, increasing the number of apoptotic cells and cognitive impairment followed by a marked increase in oxidative stress in the brain (Wang et al. 2010). In addition, patients with diabetes have higher levels of plasma concentrations of pro-inflammatory cytokines such as IL-6 and TNF-α, where they can potentiate inflammation. With significant advances made in our knowledge, it is possible to understand the mechanisms involved in the origin of brain complications so that new treatment modalities are being explored (Edwards et al. 2008). Thus, recent strategies for the prevention and treatment of complications resulting from diabetes have been studied. Insulin and hypoglycemic agents are still the most used treatments. However, current research with an experimental model of diabetes points to new antioxidants as a therapeutic approach for the prevention and treatment of neurological damage (Louzada & Vargas 2015). Several studies point to melatonin as an antioxidant and a free radical scavenger, as it stimulates the activity of antioxidant enzymes such as superoxide dismutase and glutathione reductase (El-Sokkary et al. 2003).

This hormone also plays an important role in the neuroprotection of several neurodegenerative disorders whose pathogenesis involves reactive oxygen species (Baydas et al. 2001), in addition to having anti-inflammatory effects and modulating the tumor necrosis factor-alpha (TNF-α) and the interleukin 6 (IL-6) (Hernandez-Velázquez et al. 2016). We then tested the hypothesis that exogenous melatonin may decrease or prevent the effects of diabetes on the frontal cortex of the rat brain.

MATERIALS AND METHODS

Experimental rats. The experiment was carried out at the "Laboratório de Histologia" of the "Departamento de Morfologia e Fisiologia Animal" of the “Universidade Federal Rural de Pernambuco” (UFRPE). Fifty 60 days old, virgin, albino rats (Rattus norvegicus albinus) of the Wistar lineage, weighing approximately 250g, were used The rats came from the vivarium of the UFRPE. The animals were kept in cages with food and water ad libitum, remaining in standard conditions of ±22°C with a light period between 6 a.m. - 18 p.m., divided into the following groups: control group (GC) = rats without diabetes induction, diabetic group (GD) = streptozotocin diabetes-induced rats, GD1 = streptozotocin diabetes-induced rats treated with insulin, GDMI = streptozotocin diabetes-induced rats treated with melatonin, GDMI = streptozotocin diabetes-induced rats treated with melatonin and insulin simultaneously. The experimental protocol was approved by the institutional Ethics Committee, protocol no. 36/2017.

Experimental rats. Diabetes was induced by intraperitoneal administration of a streptozotocin solution (Sigma Chemical Co., USA) after a 14-hour fast. Streptozotocin was diluted in 10mM sodium citrate buffer and pH 4.5, in a single dose of 60mg/kg of animal weight. GC group rats received equivalent doses of saline solution in the same way, and after 30 minutes of treatment, all rats were fed typically (Dall’Ago et al. 2002). Diabetes diagnosis was confirmed after seven days of streptozotocin administration. Only rats that had blood glucose above 200mg/dL (Glucometer Kit Accu-Chek Activ) were included in the study (Spadella et al. 2005). The fasting glycemia was monitored during the experimental period on days 0 (before induction), 7 (confirmation of diabetes), 15, and 30 days after administration of melatonin or insulin.

Melatonin treatment. Melatonin (Sigma, St. Louis/MO, USA) treatment was carried out for 30 days. Melatonin (10mg/kg) was dissolved in ethanol and diluted in saline and added to drinking water. The 700mL bottles were covered with aluminum foil and placed in the cages at the beginning of the night 1039 (6 p.m.) and removed the following morning (6 a.m.). During the day, water was restricted.

Insulin treatment. Insulin was administrated subcutaneously for 30 days, at a dose of 5U/dia, with two units of insulin at 10 a.m. and three remaining units at 7 p.m. (Pinheiro et al. 2011).

Weighing of animals and their brains. The rats in the experimental groups were weighed daily in the course of the experiment. After 30 days of treatment, the animals were euthanized with ketamine hydrochloride (80mg/kg) and xylazine (6mg/kg), intramuscularly, associated with 100mg/kg intraperitoneal thiopental. Subsequently, the brains were removed and weighed on an analytical scale. Frontal cortex fragments were fixed in 10% buffered formaldehyde and processed for inclusion in paraffin.

Histology and Histopathology. Cross-sections of the frontal cortex were stained for histopathology with hematoxylin and eosin.
(HE) for routine analysis and periodic acid-Schiff stain (PAS) for glycogen evaluation. The glycogen content was quantified through images captured using a Sony®1061 Video camera, coupled to the Olympus® 1062 Bx50 microscope, which was submitted to the Gimp 2.0 application to elaborate an RGB (Red-Green-Blue) histogram. (Oberholzer et al. 1996).

**Morphometry.** We evaluated three slides per animal/group. The estimate of volume [V (ref)] of the cortex was by the Cavalieri Method (Gundersen et al. 1988) the one of the neurons' numerical density (Nv) in the cortex by a previously described method (Gundersen et al. 1988, Villeda-Hernández et al. 2006). The estimate of the total numbers of neurons (N) was calculated using the following formula: N = V (ref) - Nv (Korbo et al. 1990, West 1993). In the white matter, a morphometric evaluation of the percentage of nerve fibers was performed using a 10x eyepiece containing a 25-point Waibel reticulum internally (Weibel et al. 1966) and a 40x objective, where six fields were counted, all at random and clockwise, taking into account only the points that affected the fibers. In total, 150 points were counted per animal totaling 450 points per group.

**Immunohistochemistry (IL-6 and TNF-α).** The expression of inflammatory cytokines was determined using antibodies to IL-6 and TNFα (Santa Cruz Biotechnology, Inc.) at a 1:30 dilution. The slides were deparaffinized and rehydrated in xylohol and alcohol. The antigens recovery was carried out using a solution of citrate buffer (pH 8.0) at a high temperature in the microwave for 5 minutes. Endogenous peroxidase was inhibited by a solution of hydrogen peroxide (3%) in methanol. The nonspecific antigen-antibody reaction was blocked by incubating the slides in PBS and 5% bovine serum albumin (BSA) for one hour. All antibodies (Santa Cruz Biotechnology Inc., Santa Cruz/CA, USA) were diluted in PBS/BSA 1% for one hour. Subsequently, the slides were treated with the secondary antibody for thirty minutes. The antigen-antibody reaction was observed through a brown precipitate after applying 3-3-diaminobenzidine (DAB, DakoCytomationTM) (±20 min), and counterstained with hematoxylin for 20 to 30 seconds. Then, the sections were washed in running water, dehydrated in increasing alcohol concentrations, and placed in xylol to be assembled and observed under a light microscope. The apoptotic index was determined by counting the percentage of positive cells from at least 500 nuclei subdivided into ten fields chosen at random using the 40X objective (Wu et al. 2013).

**Statistical analysis.** The following parameters were statistically analyzed: morphometry, animal weight, brain weight, glycogen quantification, apoptotic index, TNFα, and IL-6 expression. The method used was Ruskal-Wallis non-parametric method with Dunn's posthoc (P<0.05).

### RESULTS

**Glycemic levels**

Before the process of inducing diabetes, all animals in the experimental groups had blood glucose levels below 120mg/dL, with no significant differences. At seven days, all groups, except for control, had blood glucose above 300mg/dL. After 15 and 30 days of treatment, only the animals in the diabetic group (GD) continued to have a very high mean glucose (474mg/dL), while the groups treated with insulin (GDI), melatonin (GDM), and melatonin/insulin (GDMI) exhibited glucose values statistically similar to the control group (GC) (Table 1).

**Weight of rats and their brains**

There was progressive weight loss in the GD groups of rats compared to the control and the other treated groups. The treated groups (GDI, GDM, and GDMI) also showed weight reduction compared to the control, but without significant differences (Table 2). In analyzing the weight of brains from rats of the experimental groups, the GD rats showed a significantly lower value compared with other groups (Table 2). The groups treated with insulin (GDI), melatonin (GDM), and melatonin/insulin (GDMI) showed no relevant differences when compared to the GC group (Table 3).

**Histopathology and histochemistry**

In the cortical region, histopathological, there was no degeneration, atrophy, or neuronal vacuolization. However, in the white matter, a significant number of regions showed several nerve fibers devoid of axons in animals in the GD group (Fig.1). Histochemical analysis by PAS revealed a significant reduction in glycogen in animals’ cortex in the

| Table 1. Serum glucose levels (mg/dL) in rats the control and experimental groups |
|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Groups | Day 0 Induction | Day 7 Confirmation | Day 15 After induction | Day 30 After induction |
|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| GC     | 960.75 ± 5.17a          | 99.17 ± 2.14b           | 102.30 ± 3.47b           | 110.80 ± 7.46b           |
| GD     | 95.00 ± 3.36a           | 349.06 ± 12.02a         | 348.80 ± 11.43a          | 474.00 ± 9.87a           |
| GDI    | 92.25 ± 4.38a           | 338.77 ± 9.43a          | 110.30 ± 7.59b           | 128.30 ± 10.23b          |
| GDM    | 92.00 ± 3.55a           | 343.91 ± 7.88a          | 117.50 ± 9.80b           | 120.30 ± 8.16b           |
| GDMI   | 91.64 ± 3.09a           | 335.48 ± 11.22a         | 108.44 ± 8.12b           | 119.58 ± 7.39b           |
| P      | 0.0763                  | 0.0223                  | 0.0066                   | 0.0132                   |

*Means followed by the same letter in the columns do not differ significantly by Dunn’s test (p<0.05); GC = control group, GD = diabetic group, GDI = diabetic group treated with insulin, GDM = diabetic group treated with melatonin, GDMI = diabetic group treated with insulin and melatonin.*
Table 2. Weight (mg/dL) of the rats in the control and experimental groups

| Groups | Day 0 Induction | Day 7 Confirmation | Day 15 After induction | Day 30 After induction |
|--------|-----------------|--------------------|------------------------|------------------------|
| GC     | 245.30 ± 7.54a  | 273.80 ± 2.50a     | 284.50 ± 19.82a        | 305.50 ± 6.85a         |
| GD     | 247.80 ± 9.28a  | 202.50 ± 7.72c     | 242.00 ± 10.03b        | 118.00 ± 3.55c         |
| GDI    | 246.30 ± 6.39a  | 241.30 ± 11.59b    | 235.00 ± 10.98b        | 255.30 ± 9.81b         |
| GDM    | 240.00 ± 7.07a  | 237.30 ± 8.84b     | 238.50 ± 17.82b        | 259.30 ± 3.20b         |
| GDMI   | 239.80 ± 5.56a  | 243.00 ± 3.74b     | 242.00 ± 10.03b        | 118.00 ± 3.55c         |

P value 0.4242 0.0101 0.0323 0.0004

Means followed by the same letter in the columns do not differ significantly by Dunn’s test (p<0.05); GC = control group, GD = diabetic group, GDI = diabetic group treated with insulin, GDM = diabetic group treated with melatonin, GDMI = diabetic group treated with insulin and melatonin.

Table 3. Weight (g) of the brains from rats the control and experimental groups

| Groups | GC/ GD/ GDI/ GDM/ GDMI | P value |
|--------|-------------------------|---------|
|        | 1.29 ± 0.07a            | 1.10 ± 0.02b | 1.35 ± 0.05a | 1.31 ± 0.04a | 1.32 ± 0.01a | 0.0112 |

Means followed by the same letter in the columns do not differ significantly by Dunn’s test (p<0.05); GC = control group, GD = diabetic group, GDI = diabetic group treated with insulin, GDM = diabetic group treated with melatonin, GDMI = diabetic group treated with insulin and melatonin.

Fig. 1. Photomicrograph of white matter of the brain from the rats of the experiment. (A) Control group (GC), (B) diabetic group (GD), (C) diabetic group treated with insulin (GDI), (D) diabetic group treated with melatonin (GDM) and (E) diabetic group treated with insulin and melatonin (GDMI). Note in (A), (C-E) predominance of nerve fibers (arrowhead), in (B) scarce nerve fibers suggesting a degenerative process. HE, obj.20x. Insert: marked neuronal vacuolization. Nerve fibers without axon (arrows). HE, obj.20x. (F) Percentage of nerve fibers. Means followed by the same letter do not differ significantly from each other by Dunn’s test (p<0.05).

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Fig. 2. Photomicrograph of the cerebral cortex of animals in the experimental groups. (A) Control group (GC), (B) diabetic group (GD), (C) diabetic group treated with insulin (GDI), (D) diabetic group treated with melatonin (GDM) and (E) diabetic group treated with insulin and melatonin (GDMI). PAS, obj.20x. (F) Pixel quantification of glycogen content. Note a significant reduction in the GD group. Means followed by the same letter do not differ significantly by Dunn’s test (p<0.05).

GD group compared to the other groups. The animals in the groups treated with melatonin associated or not with insulin showed characteristics similar to those observed in the control animals (Fig.2).

**Morphometry**

The quantification of neurons in the frontal cortex of the brain of animals in the experimental groups revealed a significant reduction in animals in the GD group compared to the other groups. There were no significant differences between the GC and those treated with melatonin with or without insulin (Fig.3).

**Immunohistochemistry (IL-6, TNF-α) and apoptosis**

Immunohistochemistry for IL-6 revealed that the animals in the diabetic group (GD) had a strong marking in the cerebral cortex (Fig.4B). However, in the control group (GC) (Fig.4A), treated with insulin (GDI) (Fig 4C), melatonin (GDM) (Fig.4D), and melatonin/insulin (GDMI) (Fig.4E) showed a similar marking, with no significant difference between these groups (Fig.4F). For the TNF-α, the GC (Fig.5A), GDI (Fig.5C), GDM (Fig.5D), and GDMI (Fig.5E) had an equivalent mark, showing no significant difference. On the other hand, the GD (Fig.5B) showed a strong marking in the cortical region, differing significantly from the other groups (Fig.5F). The TUNEL test revealed a high rate of apoptosis in the GD group's cerebral cortex compared to animals in the other groups (Fig.6).

![Fig.3. Estimation of the number of neurons in the cerebral cortex of animals in the experimental groups. Control group (GC), diabetic group (GD), diabetic group treated with insulin (GDI), diabetic group treated with melatonin (GDM), diabetic group treated with insulin and melatonin (GDMI). Means followed by the same letter do not differ significantly from each other by the Dunn test (p<0.05).](image)

**DISCUSSION**

Regarding glycemic values, studies indicate that melatonin does not reduce hyperglycemic values in a state of diabetes (Cam et al. 2003). However, research on experimental models suggests that there is a positive relationship between melatonin and insulin. Sartori et al. (2009) showed that melatonin increased insulin sensitivity, in addition to stimulating the secretion of this hormone. This corroborates our findings that the animals in the group treated with melatonin and insulin had glucose values similar to those in the control group.

It is known that the loss of muscle mass is a characteristic of the diabetic state. This is believed to be the result of a change in protein metabolism in the state of hyperglycemia, in which this can be characterized by an increase in the catabolism of proteins and fats, leading to a decrease in the animals' body mass (Luciano & Mello 1998, Moura et al. 2012), which may justify the results observed in animals in the diabetic group. On the other hand, the animals in the groups treated with insulin associated or not with melatonin also showed weight loss in relation to the animals in the control group, but with less intensity than those in the diabetic group. This demonstrates that both insulin and melatonin can regulate body weight through energy balance, in which all energy captured by food is used and stored as energy storage (Sbem 2017).

The brain is highly dependent on glucose and hyperglycemia (Yamada et al. 2002, Bluront-Jonesa et al. 2009), and disorders of neuronal glucose transport and metabolism in hyperglycemia can induce an increased number of free radicals in diabetic conditions and subsequently negatively affect the production of the brain-derived neurotrophic factor (BDNF). BDNF plays an essential role in the survival of neurons, their growth (axons and dendrites), and synapses' formation and function (Yamada et al. 2002, Bluront-Jonesa et al. 2009). This could explain the axonal degeneration of the in white matter and reduced levels of glycogen in the cortex of the diabetic rats, which was prevented in animals treated with melatonin regardless of the association with insulin, probably due to the ability of this indoleamine to promote the expression of BDNF (Luo et al. 2017). Concerning insulin, it was reported (Ghasemi et al. 2013), the presence of more significant numbers of receptors for this hormone in several sites such as the olfactory bulb, cerebral cortex, hippocampus, cerebellum, and choroid plexus, suggesting its importance in neuronal survival, synaptic plasticity and glucose absorption (Banks 2004).

There was a reduction in the number of neurons in the rats' frontal cortex from the diabetic group without treatment. This is undoubtedly related to the high apoptotic index observed in these animals, explaining the reduction in brain weight concerning rats in the other groups. Apoptosis is a series of processes programmed to carry out cell death and plays a significant role in maintaining tissue homeostasis. When deregulated, this process is associated with several pathological states such as neurodegenerative diseases and diabetes mellitus (Lee & Pervaiz 2007, Dorsemans et al. 2017).

In previous studies related to the central nervous system, the administration of exogenous melatonin resulted in a decrease in TUNEL-positive cells, indicating the neuroprotective effect of this hormone and suggesting that melatonin could act in the prevention of neurodegenerative diseases by inhibiting the intrinsic pathway of apoptosis (Lima et al. 2005, Tuzcu & Baydas 2006, Ferreira et al. 2010). Regarding insulin, it can...
Fig. 4. Immunohistochemistry for IL-6 in the brain of the rats. Observe in (A) control group (GC), (C) diabetic group treated with insulin (GDI), (D) diabetic group treated with melatonin (GDM) and (E) diabetic group treated with insulin and melatonin (GDMI) weak marking, and in (B) diabetic group (GD) strong marking in the cortical layer. (F) Quantification in pixels. Note a significant increase in the GD group. Means followed by the same letter do not differ significantly by Dunn’s test ($p<0.05$).
Fig. 5. Immunohistochemistry for TNF-α in the brain of the rats. Observe in (A) control group (GC), (C) diabetic group treated with insulin (GDI), (D) diabetic group treated with melatonin (GDM) and (E) diabetic group treated with insulin and melatonin (GDMI) weak marking, and in (B) diabetic group (GD) strong marking in the cortical layer. (F) Quantification in pixels. Note a significant increase in the GD group. Means followed by the same letter do not differ significantly by Dunn’s test ($p<0.05$).
Fig. 6. Immunohistochemistry for apoptosis in the brain. Observe in (A) control group (GC), (C) diabetic group treated with insulin (GDI), (D) diabetic group treated with melatonin (GDM) and (E) diabetic group treated with insulin and melatonin (GDMI) weak marking, and in (B) diabetic group (GD) strong marking in the cortical layer. (F) Apoptotic index. Note a significant increase in the GD group. Means followed by the same letter do not differ significantly by Dunn’s test (p<0.05).
inhibit apoptosis in situations of oxidative stress, ischemia, and toxicity of the β-amyloid peptide (Ghasemi et al. 2013).

Pro-inflammatory cytokines play a crucial role in the pathogenesis of diabetes (Ghosh et al. 2015). Our results indicate a strong marking of IL-6 and TNF-α in the cortical area of the rats’ frontal brain in the diabetic group compared to control rats in the other groups. This points out to a neurodegenerative process that can compromise brain functions (Wajant et al. 2003, Cutando et al. 2015). However, these effects were prevented by melatonin with or without insulin, indicating this hormone is a potent inhibitor of inflammatory interleukins (Kumar & Sharma 2010, Seraphim et al. 2000). Other studies link the binding of insulin to the regulation of the inflammatory response since this hormone participates in processes that inhibit the production of inflammatory cytokines such as IL-6 and TNF-α.

**CONCLUSION**

The treatment with melatonin associated with insulin proved to be beneficial in preventing the effects of diabetes on the rat brain’s frontal cortex. However, this administration’s clinical and physiological importance requires further clarification to understand better the mechanisms by which melatonin exerts these beneficial effects.

**Conflict of interest statement.** The authors have no conflicts of interest to declare.

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