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KELULUT HONEY SUPPLEMENTATION PREVENTS SPERM AND TESTICULAR OXIDATIVE DAMAGE IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Graphical abstract

Abstract

Previous studies found that Kelulut Honey produced by *Trigona spp.* bees is able to prevent oxidative damage in various pathological conditions. Thus, the present study aimed to determine whether Kelulut Honey could prevent the sperm and testicular damage in streptozotocin-induced diabetic rats. Male Adult male Sprague-Dawley rats were divided into four groups: Non-Diabetic (NDM), Non-Diabetic with Kelulut Honey supplementation (NDMKH), Diabetic without supplementation (DM) and Diabetic with Kelulut Honey supplementation (DMKH). Kelulut honey was given at the dose of 2.0 g/kg weight daily via gavage for 28 consecutive days. Results showed that sperm quality produced by diabetic rats supplemented with Kelulut honey significantly improved compared to the diabetic control groups ($p<0.05$). SOD activity and GSH level increased significantly ($p<0.05$) whereas PC and MDA levels significantly decreased in sperm and testis of DMKH rats when compared to DM rats ($p<0.05$). Histological observation showed obvious increase in spermatozoa in the lumen of epididymis and increased spermatogenic cells density in the testis of DMKH group. In conclusion, Kelulut Honey has a potential in preventing the damage of sperm and testis in diabetic rats.

Keywords: Kelulut honey, reproductive system, diabetes mellitus, testis, sperm, oxidative stress

Abstrak

Kajian terdahulu mendapati madu Kelulut yang dihasilkan oleh lebah *Trigona* spp. dapat mencegah kerosakan oksidatif dalam pelbagai keadaan patologi. Oleh itu, kajian ini dijalankan bagi mengenal pasti keupayaan madu Kelulut dalam mencegah kerosakan sperma dan testis pada tikus diabetis aruhan streptozotosin. Tikus jantan Sprague-Dawley dibahagikan kepada empat kumpulan; Bukan Diabetis (NDM), Bukan Diabetis dengan suplementasi Madu Kelulut (NDMKH), Diabetis (DM) dan Diabetis dengan suplementasi Madu Kelulut (DMKH). Madu Kelulut diberikan pada dos 2.0 g/kg berat badan setiap hari secara gavaj oral selama 28 hari berturut-turut. Hasil kajian menunjukkan bilangan, motiliti dan viabiliti sperma meningkat manakala peratus morfologi abnormal sperma pula menurun secara signifikan pada tikus diabetis yang diberikan suplementasi madu Kelulut ($p<0.05$). Aktiviti SOD dan GSH meningkat dengan signifikan ($p<0.05$) manakala aras PC dan MDA menurun secara signifikan pada testis dan sperma tikus kumpulan DMKH berbanding kumpulan DM ($p<0.05$). Pemerhatian histologi juga menunjukkan bilangan sperma yang lebih tinggi di dalam lumen epididimis dan peningkatan sel-sel spermatogenik pada testis kumpulan DMKH berbanding DM. Kesimpulannya, Madu Kelulut didapati berpotensi untuk mencegah kerosakan sperma dan testis akibat diabetes.

Kata kunci: Madu Kelulut, sistem reproduktif, diabetes melitus, testis, sperma, tekanan oksidatif

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1.0 INTRODUCTION

Prolonged state of hyperglycaemia in diabetes mellitus condition eventually leads to the formation of free radicals, especially the reactive oxygen species (ROS) [1]. In normal condition, the excess free radicals will be taken up by the antioxidant systems. Antioxidants would deplete in pathological conditions, causing the free radicals to present in excess amount. Free radicals have the ability to interact with macromolecules such as nucleic acids, lipids, proteins and carbohydrates, which would leading to oxidative stress condition thus causing damaging effects [2]. The generated oxidative stress will then affect the male reproductive function by disturbing the steroidogenic capacity and altering the germinal epithelium's capacity to differentiate spermatozoa, therefore producing abnormal sperm [3]. The factor that made the testis and mammalian sperm cells especially vulnerable to oxidative stress is they are mainly made up of polyunsaturated fatty acids (PUFA), thus putting them at a higher risk of oxidative damage by peroxidation [4].

Recently, more studies have been made focusing the benefits and advantages of honey on the reproductive system especially in normal health condition. The antioxidant content in honey is proven to have the ability to prevent and reduce testicular damage and lipid peroxidation in rats exposed to cigarette smoke [5]. Honey supplementation to rats increases epididymal sperm count besides increasing testicular enzyme markers for spermatogenesis [6]. Meanwhile, Syazana *et al*. [7] showed that supplementation of honey improved motility and morphology of sperm in normal rats, showing its ability to enhance fertility.

Kelulut honey (KH) is produced by a stingless bee species, *Trigona* spp. and is physically different from honey produced by common honey bee (Apis sp.) where it is more darker in colour and more runny in consistency [8]. Previous study on KH showed that it possessed antibacteria and antiaging properties [8, 9]. To date, research on the effects of KH supplementation to male reproductive system is still not done. Therefore, this study was conducted to determine the effects of oral administration of Malaysian KH produced by *Trigona* spp. bee for 28 days on the testicular and sperm damage in streptozotocin-induced diabetic rats.

2.0 METHODOLOGY

2.1 Preparation of Kelulut honey

Pure KH is obtained from Integrated Trigona Bee Honey Marketing & Distribution, Bandar Baru Bangi, Selangor, Malaysia. Honey is kept at room temperature and shaken well before used.

2.2 Animals

Thirty-two male *Sprague-Dawley* rats weighing between 230-250 g were obtained from the Animal Unit of Universiti Kebangsaan Malaysia which is located at Kolej Tun Syed Nasir, Jalan Temerloh, 53200, Kuala Lumpur, Malaysia. All the animal handling protocols were performed in accordance to the guidelines issued by the UKM Ethics Committee (UKMAEC No: FSK/BIOMED/2014/JAMALUDIN/26-NOV/629-DEC-2014-JUNE-2015). The rats were acclimatized to the laboratory environment for a week. They were kept 2 animals per cage, at room temperature with 12 hour light/dark cycle and had free access to commercial pellet and water *ad libitium* throughout the research period.

2.3 Diabetes Induction and Treatment

The rats randomly distributed into four groups of 8 rats each. The four groups were the normal group (NDM), normal rats treated with kelulut honey group (NDMKH), diabetes group (DM), and the diabetic group treated with kelulut honey (DMKH). Diabetes was induced in groups DM and DMKH via single intraperitoneal injection of STZ (Sigma Chemicals, ST. Louis, Missouri, USA) (freshly dissolved in normal saline) at a dose of 65 mg/kg body weight after overnight fasting [10]. The fasting blood glucose level was measured (using Accu-Chek Glucometer, Roche, Germany) 3 days later and the rats with the blood glucose level of 15 mmol/L or more were considered to be diabetic and included in the study. Kelulut honey at the dose of 2.0 g/kg was given to NDKH and DMKH group whereas NDM and DM group only received normal saline. The dosage was calculated based on human intake. The treatments were given orally through force feeding for a duration of 28 days.

2.4 Sample Collection and Preparation

After 28 days of treatment, the rats were sacrificed after being fasted overnight. The male reproductive organs, which were the testis and the epididymis were taken out for weighing, biochemical analysis as well as histological observations. Testis homogenate was prepared by mixing the seminiferous tubules in homogenate buffer at 20% (w/v) ratio. The homogenate was then homogenized and centrifuged at 8000 × g for 20 minutes at 4°C. The supernatant was extracted and kept at 80°C and was used for biochemical analysis.

The cauda part of the epididymis was placed in 2 ml of Hank's Buffered Saline Solution (HBSS) and was mechanically minced by using anatomical scissors. The suspension was stirred gently and left for five minutes to allow the sperm to leak out. A part of the sperm suspension was used for sperm characterization analysis while the remaining was kept at -80°C prior biochemical analysis.

2.5 Sperm Characteristics Analysis

The sperm was collected as quickly as possible after dissection. 10 µl of homogenous sperm suspension was placed on a "Makler Counting Chamber" (Sefi-Medical Instruments Sdn. Bhd., St. Santa Ana, CA, USA) and epididymal sperm count and motility were determined under a light microscope at 100x magnification. Sperm count was expressed as milion sperm cells per ml of suspension while sperm motility in percentage of motile sperm.

For sperm viability assessment, 10 µl of sperm suspension was added with 10 µl eosin-nigrosin stain and thick smear were done on the slides. Normal live sperm exclude the eosin stain and appear white in colour, whereas "dead" sperm take up eosin and appear pinkish. The percentage of abnormal sperm morphology was calculated using a thin smear of sperm suspension. The dried smear was stained with Diff-Quik staining kit. Two hundred sperm were examined per slide to measure the morphological abnormalities under oil immersion. The data are presented as the percentage of abnormal sperm morphology.

2.6 Determination of Malondialdehyde (MDA) Level

The level of MDA was measured based on the method described by Stocks and Dormandy [11]. Peroxidation of lipid was measured by the reaction between MDA in the sample and thiobarbituric acid (TBA) that will form pink adducts of MDA-TBA complex. This chromogen can be measured spectrophotometrically at 532 nm. The results were expressed as nmol/mg protein.

2.7 Determination of Protein Carbonyl (PC)

The level of PC was measured according to the method described by Levine *et al*. [12] as an indication for protein oxidation. The reaction between 100 µl of sample and 2,4-dinitrophenylhydrazine (DNPH) form protein-hydrazones conjugates that can be measured spectrophotometrically at 375 nm. The results were expressed as nmol/mg protein.

2.8 Determination of Superoxide Dismutase (SOD) Enzyme Activity

SOD activity was evaluated by its ability to inhibit the ferricytochrome reduction according to the method by Beyer and Fridovich [13]. 20 µl of sperm homogenate was mixed with substrate mixtures (PBS-EDTA, L-methionine, NBT.2HCl, Triton-X) and incubated in an aluminium box under 20 watt lamp for 7 minutes. Colour development was measured at 560 nm, and the activity of SOD was expressed as units of E/mg protein, with one unit of enzyme inhibiting 50% of nitro blue tetrazolium (NBT).

2.9 Determination of Glutathione (GSH) Enzyme Activity

The activity of GSH in the sample is based on the reaction between 5, 5'- dithiobis acid [2-nitrobenzoic] (DTNB) and GSH molecule [14]. DTNB will bind to GSH molecule and form 5-thio nitrobenzoic acid (TNB) and GS-TNB. TNB molecule, which is yellow in colour, can be measured using spectrophotometer at 412 nm. The results were expressed as mmol/mg of protein.

2.10 Organ Histology

The testis and epididymis were processed in a series of step that includes dehydration, washing, impregnation and embedding. Then the tissue was sliced using a microtome at 5 µm thickness. After that, the tissue was transferred to the slide and being dried before stained using Hematoxylin and Eosin (H&E) technique which is a standard histological staining [15]

2.11 Statistical Analysis

Shapiro-Wilk test was used for normality testing on all data ($p>0.05$) while Levene's test was used to assess homogeneity ($p>0.05$). By using SPSS Version 22, the differences between all four groups were compared using one-way analysis of variance (ANOVA). Differences between groups were statistically significant at $p<0.05$ and all results are expressed as the mean ± standard error of the mean (SEM).

3.0 RESULTS AND DISCUSSION

3.1 Reproductive Organ Weight

Table 1 shows the weights of reproductive organs; testis and epididymis in the experimental groups. The testicular weight significantly increased in Kelulut honey-supplemented non-diabetic group when compared to the non-diabetic group ($p<0.05$) whereas the increase in epididymal weight is not significant. The testicular and epididymal weights in diabetic control rats are significanly lighter when compared to non-diabetic control group ($p<0.05$). The supplementation of Kelulut honey to diabetic rats showed to increase both testicular and epididymal weights but the difference is not significant

Testis weight is dependent on the mass of the differentiated spermatogenic cells therefore the reduction in the testis weight may be due to the decreased density of germ cells and spermatogenic arrest. In concert to previous report, our study showed that diabetic rats had significantly low sperm quality [16]. The generation of oxidative stress during early diabetic phase is likely to contribute to the impairment of the spermatogenesis process [17].

Table 1 The effect of diabetes and Kelulut honey supplementation on testicular and epididymal weights (g) in rats

Group	Weight (g)	
	Testes	Epididymis
NDM	1.044 ± 0.1	0.358 ± 0.02
NDMKH	1.184 ± 0.04[a]	0.368 ± 0.03
DM	0.584 ± 0.06[a]	0.236 ± 0.02[a]
DMKH	0.791 ± 0.06	0.313 ± 0.02

The values are expressed as mean ± SEM
[a]Significant difference as compared with the NDM control group at P<0.05.

3.2 Sperm Characteristics

The sperm characteristics of the experimental groups are shown in Table 2. Administration of Kelulut honey significantly increased the epididymal sperm count, motility and viability in non-diabetic rats when compared to the non-diabetic control group (p<0.05), showing a possible property of fertility enhancer in Kelulut honey. Significant decreases were showed in all sperm characteristics observed in the sperm of diabetic group compared to that of the non-diabetic group (p<0.05). Kelulut honey showed the potential to increase the sperm motility, viability and lowers the percentage of abnormal sperm morphology in Kelulut honey-treated diabetic group compared with the diabetic control group (p<0.05).

Sperm are made up of high polyunsaturated fatty acid (PUFA) in composition, which make them highly susceptible to oxidative damage. Disruption of spermatozoa membrane matrix structure due to lipid peroxidation may contribute to deterioration of membrane integrity thus contributing to the increase in abnormal morphology of sperm. In addition, damage of the membrane causes disruption to its fluidity, leading to motility loss [18]. The reduction of sperm count was consistent with the depletion in the epididymal weight.

KH supplementation to diabetic rats has shown to increase sperm quality, exhibiting its protective effects against disturbance to spermatogenesis process in diabetic condition. Kek *et al*. [19] reported that KH contains higher phenolic content as well as having the highest antioxidant level of all honeys tested in their study including Tualang honey and Gelam honey. The high antioxidant level in the Kelulut honey might balanced out the high free radicals generated in the reproductive organs, rendering the spermatogenesis process back to normal.

3.3 Estimation of MDA, PC, SOD and GSH Levels in Testis and Sperm

Table 3 shows that MDA and PC levels in both testis and sperm were significantly increased (p<0.05) in diabetic rats when compared to normal control. Kelulut honey-treated diabetic rats showed significantly lowered MDA and PC levels compared to the diabetic control group (p<0.05). Moreover, the SOD and GSH levels were also significantly decreased in diabetic rats (p<0.05) when compared to the normal control. Supplementation of Kelulut honey to diabetic rats showed to assist in increasing the SOD and GSH levels significantly (p<0.05) as compared to the SOD and GSH levels in the testis and sperm of diabetic control. The improvement is comparable to the normal control rats as well, showing amelioration of the injuries towards normal condition.

Chronic hyperglycaemia in diabetic patients is found to be the cause of increased oxidative stress in the body [20]. Oxidative stress has previously proven to be responsible for the initiation and progression of diabetic complications [21]. Significantly high levels of MDA and PC, which reflects lipid and protein peroxidation respectively [22, 23], were observed in the testes and sperm of diabetic rats without KH supplementation in the current study. On top of that, activity levels of SOD and GSH were found to be significantly low in the diabetic control rats. The elevated oxidative stress in diabetic condition might have hamper the SOD and GSH activity besides increasing the levels of MDA and PC. These results were in line with previous study that found that STZ-induced diabetic condition induced oxidative stress in the renal in rat [24].

Table 2 Effects of diabetes and Kelulut honey supplementation on total caudal sperm count, sperm motility, viability and morphology

Group	Total Sperm Count (10^6/ml)	Sperm Motility (%)	Sperm Viability (%)	Abnormal Morphology (%)
NDM	27.6 ± 3.28	43.09 ± 3.91	34.3 ± 2.20	7.74 ± 0.48
NDMKH	38.8 ± 3.94[a]	63.88 ± 3.03[a]	47.9 ± 3.95[a]	7.12 ± 0.68
DM	13.1 ± 2.37[a]	18.71 ± 3.28[a]	17.1 ± 1.43[a]	20.91 ± 2.07[a]
DMKH	16.6 ± 1.92[a]	37.86 ± 2.90[b]	29.9 ± 2.76[b]	12.39 ± 0.88[a]

The values are expressed as mean ± SEM
[a]Significant difference as compared with the NDM control group at P<0.05. [b]Significant difference as compared with the DM group at P<0.05.

Table 3 Effects of supplementation of Kelulut honey on MDA, PC, SOD activity and GSH levels in testes and epididymal sperm in the experimental groups

	Testis				Sperm			
	MDA	PC	SOD	GSH	MDA	PC	SOD	GSH
NDM	7.43±0.1	035±0.014	192.33±4.05	0.14±0.04	2.41 ± 0.37	15.35 ± 0.86	0.24 ± 0.04	0.26 ± 0.02
NDMKH	6.68±0.33	0.281±0.02	226.12±9.11	0.18±0.04	2.31 ± 0.16	16.77 ± 2.88	0.26 ± 0.02	0.31 ± 0.02
DM	18.75±0.15[a]	0.62±0.02[a]	87.66±9.11[a]	0.04±0.02[a]	3.93 ± 0.39[a]	29.8 ± 0.8[a]	0.10 ± 0.03[a]	0.08 ± 0.01
DMKH	15.79±0.15a[a,b]	0.59±0.01[a]	134.47±12.54[b]	0.06±0.06[a,b]	2.91 ± 0.14[b]	18.65 ± 2.19[b]	0.19 ± 0.04[a,b]	0.19 ± 0.02

MDA, PC and GSH are expressed as nmol/mg protein whereas SOD is expressed as (E/mg protein)
The values are expressed as mean ± SEM
[a]Significant difference as compared with the NDM control group at P<0.05. [b]Significant difference as compared with the DM group at P<0.05.

Supplementation of KH to diabetic rats were also found to significantly reduced the MDA and PC levels in both testis and sperm. This finding is consistent to previous studies which reported that honey and antioxidant administration has the ability to control oxidative stress [5, 25]. Increase in the SOD activity and GSH levels were also observed. The improvement to the oxidative biomarkers status was most probably due to the high phenolic content naturally found in this honey, which might contribute to the improvement by its ability to scavenge free radicals and to increase the levels of endogenous antioxidant.

3.4 Histological Observation of Testis and Epididymis

Testis

The testis (Figure 1) of DM group did show the appearances of abnormalities. Testis morphological observation of the diabetic control rat showed that the arrangement of spermatogenic cells in the germinal epithelium is disorganized where the division of spermatogenesis layers are not clear. Spermatids and Sertoli cells are not visible whereas Leydig cells present in small number. The supplementation of Kelulut honey showed improvement in the testicular degeneration induced by diabetes condition where the structure is similar to that of non-diabetic control group.

In addition to the changes in antioxidant and peroxidation status, changes in the structures of testis and epididymis can also be observed in diabetic rats. Diabetic condition causes reduction in the tubule diameter of seminiferous tubules caused by cell apoptosis and atrophy [26].

Besides that, the steroidogenic and spermatogenic capacities of testis are shown to be susceptible to superoxide radical damage [27]. Maturation arrest is observed in the present study, represented by few numbers of spermatogenic layers in the diabetic group. Our results indicate that STZ-induced diabetes caused alterations in number of Leydig cells and impairment in cell function as a result of oxidative stress as shown by the extensive loss of germ cell layers.

Epididymis

Figure 2 shows the epididymal histological observation of all groups. Diabetic control group showed empty epididymal lumens with presence of immature spermatogenesis products when compared to non-diabetic control and Kelulut honey-supplemented non-diabetic groups that are both tightly packed with spermatozoa. Kelulut honey-supplemented diabetic group showed lumens containing spermatozoa but not as packed as both non-diabetic group and NDMKH group. The morphological changes in the epididymal of DMKH group are also minimal and comparable to the non-diabetic group.

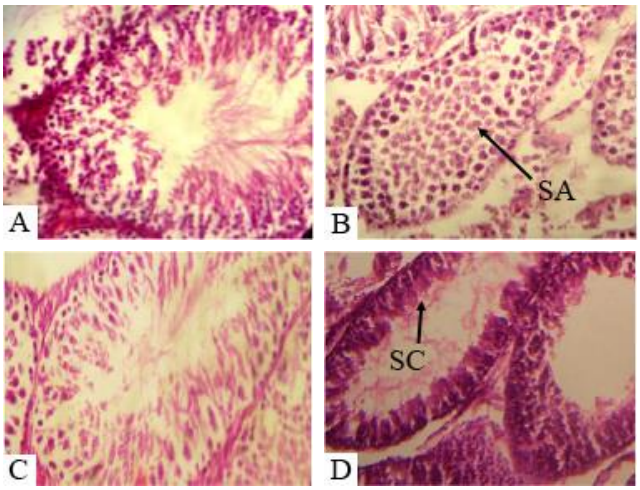

Figure 1 Testis histology of normal group ND (A), normal group treated with kelulut honey NDMKH (B), diabetic group DM (C), and diabetic group treated with Kelulut honey DMKH (D) under magnification of X400 using a light microscope. Testicular sections of DM rats showing (B) seminiferous tubules filled with spermatogenic cells up to spermatids only with no sperm formation (SA, spermatogenic arrest). DMKH (D) showing seminiferous tubules lined by few layers of spermatogonic cells (SC, spermatogonic cells)

The morphological changes in the testis were in consistent to the disturbance in spermatogenesis, seen in the reduced quality and density of the sperm produced. Reduction of sperm production in diabetic condition is supported by histological observation in the epididymis, in which there were reduction in the density of spermatozoa in the lumen of diabetic group when compared to the normal control.

The histological sections of the current study showed improvement of testicular structures as well as increase of spermatogenic cells of Kelulut honey supplemented diabetic rats when compared to the diabetic control. This shows that Kelulut honey possibly acts as physiologic modulators of spermatogenic cells proliferation which influence the cell cycle of the seminiferous epithelium, leading to increased spermatogenesis. This is also supported by the increase of sperm density and the condensed epididymal lumens in in the epididymis of Kelulut honey-supplemented diabetic group. The feeding of Kelulut honey is shown to have potential to provide protection against oxidative damage in the reproductive organs as the Kelulut honey-supplemented diabetic groups showed similar structures to that of the diabetic control group.

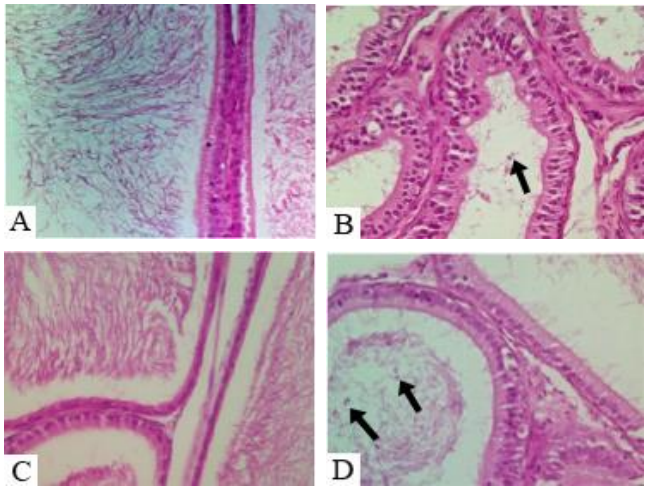

Figure 2 Epididymis of group DM (A), DM (B), NDMKH (C) and DMKH (D). The arrows show the presence of immature spermatogenesis products in the epididymal lumen of both DM (B) and DMKH (D) rats. The decrease in sperm density is observable in the epididymis of diabetic control group (B) when compared to the normal control group (A). Epididymis histology of DM rats (B) showed distortions in the linings of epididymal tubules with no presence of matured sperm. NDMKH rats (C) showed increased density of matured sperm in the tubules when compared to untreated non-diabetic rats (A). DMKH (C) showed improvement in the tubule architecture, and presence of immature and mature sperm. Sections were prepared and stained with H&E and observed with x400 magnification

4.0 CONCLUSION

Supplementation of Kelulut honey at 2.0 g/kg body weight for 28 days shows the ability to increase sperm count, percentage of motility and viability and reduce percentage of abnormal morphology significantly. The increase in SOD activity and GSH levels were observed whereas MDA and PC level shows significantly lower levels in the Kelulut honey-supplemented diabetic group when compared to the diabetic control group. The sperm density increment in the epididymis in Kelulut honey-supplemented group is also observed. Therefore, it can be concluded that Kelulut honey supplementation is able to prevent diabetes-induced testicular and sperm damage in STZ-induced rats.

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