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Effects of Dolutegravir (DTG) on Survival, Pupariation and Emergence in *Drosophila melanogaster*: The Rescue Role of *Brassica oleracea*

Amagon Leritshimwa [1,2*], Haruna Abigail Awadzi [1], Amagon Kennedy [1] Wanche Ernest Magani [1,2], Falang Kakjing Dadul [1] and Bukar Bayero Bukata [1]

[1] Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Jos, Jos, Nigeria.
[2] African Center of Excellence in Phytomedicine Research and Development, University of Jos, Jos, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

The study aimed at determining the protective role of *Brassica oleracea* on dolutegravir-induced changes in Pupariation and Emergence of *Drosophila melanogaster*. *D. melanogaster* aged 3-5 days old were exposed to different concentrations (0.5 to 4 mg/ 5 g diet) of dolutegravir and *B. oleracea* extract (7.5–1000 mg/5 g diet) for 7 days to determine the lethal concentration (LC_{50}). *D. melanogaster* were then exposed to the extract (50, 100, 200, and 400 mg/5 g diet) and controls (diet alone and vitamin C) to assess their effects on pupariation and emergence. A 14-day assay was also performed to evaluate the effect of the extract and toxicant (dolutegravir) on fly survival. The result showed a dose-dependent significant decrease ($P < 0.05$) and a dose-dependent significant increase ($P < 0.05$) in survival for *D. melanogaster* exposed to dolutegravir and the extract respectively, when compared to the control group. Results showed a delay in pupariation and decrease in mean pupariation in flies exposed to dolutegravir alone. An improvement in the same parameters was observed in *D. melanogaster* pre-treated with the extract before exposure to

**Corresponding author: E-mail: leritshimwa@yahoo.co.uk, amagonl@unijos.edu.ng;*

dolutegravir. *D. melanogaster* pre-treated with 200 and 400 mg extract per 5 g diet showed emergence that was comparable to those in the control groups. A significant decrease ($P < 0.05$) was observed in the groups exposed to 50 and 100 mg extract per 5 g diet, suggesting no protection at these doses. This study concludes that *B. oleracea* leaf extract, at certain concentrations, is able to protect against dolutegravir-induced changes in pupariation and emergence in *D. melanogaster*.

Keywords: Brassica oleracea, dolutegravir, drosophila, emergence, Pupariation.

1. INTRODUCTION

D. melanogaster is a specie of fly (the taxonomic order Diptera) in the family Drosophilidae. The specie is often referred to as the fruit fly, though its common name is more accurately the vinegar fly. Starting with Charles W. Woodworth's proposal of the use of this species as a model organism, *D. melanogaster* continues to be widely used for biological research in genetics, physiology, microbial pathogenesis, and life history evolution [1]. The life cycle of *D. melanogaster* is short and completes in about three weeks. Embryonic development, which follows fertilization and the formation of the zygote, occurs within the egg membrane. The egg produces larva, which eats, grows, and at length becomes a pupa. The pupa, in turn, develops into an imago or adult. The duration of these stages varies with the temperature. *Drosophila* stock ought to be kept at room temperature where the temperature does not range below 20^{o}C or above 25^{o}C. They are bred on fermenting medium, which contains corn, dextrose, sugar, and yeast extract. Their breeding ratio is 1:3 (male: female). The male and the female are differentiated (under the microscope) by their size, markings on their abdomen and presence of sex combs following anesthetization [2]. Sexual dimorphism is characteristic of *Drosophila spp*. Therefore, males can be easily differentiated from females having differences in size and color. The females are about 2.5mm long while the male is somewhat smaller than the female, with dorsal side of the male body being darker due to a distinct black patch at the abdomen [3].

In the laboratory, *D. melanogaster* larvae typically wander and pupate on the walls of the container because the bottom of the rearing vial or bottle is usually entirely covered by food [4]. The distance that larvae pupate from the surface of the food 'pupation height' is a polygenic trait that responds effectively to bidirectional selection [5,6] and is influenced by light, temperature, humidity, pH, density, and parasitism [7-9]. When laboratory culture conditions are enriched with horizontal semi-natural arenas (soil, agarose, etc.) around the feeding medium, larvae prefer to wander and pupate in these arenas [6,10].

Dolutegravir (DTG) is a second-generation Integrase Strand Transfer Inhibitor (INSTI) used in the management of HIV1. The FDA approved DTG in August 2013 for infected patients above the age of 12years and weighing over 40kg. Since its approval 7years ago, many studies have been carried out to evaluate safety on prolonged use and its efficacy as well as the development of resistance by the virus to the drug. Studies have shown that DTG used in the management of HIV has the potential to cause oxidative stress in cells, which leads to several complications such as hepatotoxicity and other major side effects [11]. The study of DTG effect on pupariation and emergence of *D. melanogaster* might be beneficial in terms of evaluating the risk to patients placed on the drug for the management of HIV [12].

Brassica vegetables are the most important genus of the Brassicaceae family and consist of thirty-seven different species. They contain low fat, high vitamin, mineral, and fiber as well as various phytochemicals amongst which are hydrolytic products of glucosinolates which prevent oxidative stress, induce detoxification enzymes, stimulate the immune system, reduce cancer risk, inhibit malign transformation and carcinogenic mutations in addition it reduces the proliferation of cancer cell [13].

Metamorphosis in *D. melanogaster* may be divided into two stages; a 12 hours prepupal period marked by pupariation (the onset of the larval-pupal transition) and a subsequent pupal period lasting 84 hours. Pupariation is marked by a sudden release of ecdysteroid hormone secreted from the ring gland. The larval cuticle becomes the puparium or pupal case that surrounds the metamorphosing fly until it ecloses. Apolysis is the term for the retraction of the epidermis from the cuticle of the third instar larva. Once apolysis is complete, a characteristic

gas bubble forms in the prepupa abdomen, at this stage the developing pupa is able to float in water. Next, the eversion of the head takes place, approximately 12 hours from the start of pupation. The process itself is sudden, lasting about 10 minutes and orchestrated by contractions of abdominal muscles. Head eversion marks the beginning of the true pupal state. During pupariation, the marginal disc undergoes eversion to form the basic shape of the adult head, thorax, and abdomen. Wing, leg, and haltere discs fuse to form the thorax. The eye-antennal complex fuses to form a single head capsule and the head and thorax fuse with the abdomen [14].

1.1 Aim of the Study

The aim of the study was to determine the rescue role of *B. oleracea* in protecting against Dolutegravir-induced changes in pupariation and emergence of *D. melanogaster.*

2. MATERIALS AND METHODS

DTG tablets 50 mg per tablet was used in this study. A total of thirty (30) tablets of the fixed-dose formulation were weighed to determine the average weight per tablet. The tablets were pulverized using porcelain mortar and pestle. The appropriate quantities of powder that will contain the desired amount of active ingredient were calculated and weighed using analytical balance (Meltlar Model No. MT-200B).

2.1 Plant Collection, Identification and Extraction

The plant was purchased from Farin gada vegetable market Jos North Local Government area, Jos, Plateau State and the identification and authentication carried out at Federal College of Forestry and a voucher number obtained. This was extracted using water.

2.2 Animal Model

D. melanogaster (Harwich strain) at 3-5days old was obtained from the Africa Centre of Excellence in Phytomedicine Research and Development (ACEPRD), University of Jos, Jos, Nigeria. The fly stock was maintained at a temperature of 23±1°C and 60% relative humidity under 12 h dark/light cycles. The flies were fed on standard Drosophila medium composed of cornmeal (1% w/v), brewer's yeast (2% w/v), agar, and methylparaben (0.08% w/v).

2.3 Determination of LC_{50}

The determination of LC_{50} was carried out following the methods described by Mohammad & Singh, 2009 and Charpentier et al. 2014, [15,16] with slight modifications. Sixty (60) flies of age range 1- 3 days were anesthetized under ice, counted and exposed to series of graded concentrations of DTG, 0.5 mg, 1.0 mg, 2.0 mg, 3.0 mg, 4.0 mg and diet only (as control) per 5 g fly food respectively for 7 days. Observations were made at 24-hour intervals and any mortalities were recorded. Data obtained were subjected to a dose-response simulation using Graphpad prism 8.0 for LC_{50} determination. The same was done for *B. oleracea* extract at 50 mg, 100 mg, 150 mg and 200 mg per 5 g diet.

2.4 Fourteen-day Survival Assay

In this experiment, sixty (60) flies (of both sexes), aged 1-3 days old, were exposed to different doses of DTG and extract (per 5 g fly food). This was performed in five replicates for 14 days as described by Abolaji et al. 2014 [17]. The number of live and dead flies was scored daily till the end of the experiment, and the survival rate was expressed as a percentage of live flies.

2.5 Treatment for Reproductive Ability (Pupariation and Emergence)

Sixty (60) virgin *D. melanogaster* (both sexes), age range 1-3 days old, were exposed to Dolutegravir, 0.5 mg, 1.0 mg, 2.0 mg, 3.0 mg, 4.0 mg and diet only (as control) per 5 g fly food respectively per 10 g fly food as described by Abolaji et al. 2014 [17]. The same was done for the extract at 50, 100, 200 and 400 mg respectively. Negative and positive control groups were also treated.

After five (5) days of treatment, 5 male and 5 female *D. melanogaster* were randomly selected in each treatment group and transferred into fresh vials with normal food for 24 hours, and the eggs laid in each vial during this period was kept for 21 days for the emergence of adult flies. The reproductive ability of the *D. melanogaster*, after exposure to DTG and extract, was assessed by counting the number of pupae and later the number of *D. melanogaster* that emerged daily. The mean number of *D. melanogaster* that emerged gives a measure of reproductive ability.

2.6 Statistical Analysis

The results were analyzed using GraphPad Prism 8 (GraphPad Software Inc., CA).

3. RESULTS

The percentage yield was calculated to be 43.745 % and the phytochemicals present in *B. oleracea* leaf extract included Alkaloids, Saponins, Tannins, Flavonoids and Carbohydrates.

3.1 LC_{50} Determination

The LC_{50} of DTG was 2.144 mg/5 g diet while that of the extract could not be determined even at doses up to 1000 mg/5 g diet.

3.2 Survival Assays

The result showed a dose-dependent significant decrease ($P < 0.05$) in the survival of the *D. melanogaster* exposed to DTG while there was a dose-dependent significant increase in survival for *D. melanogaster* exposed to different concentrations of the extract when compared to the control group.

3.2.1 Fourteen-day survival assay for DTG

There was a significant dose-dependent decrease in percentage survival in *D. melanogaster* exposed to different doses of DTG as compared to the control group that was on diet alone with survival dropping as low as 8.3% in *D. melanogaster* exposed to 4 mg/5 g diet of DTG.

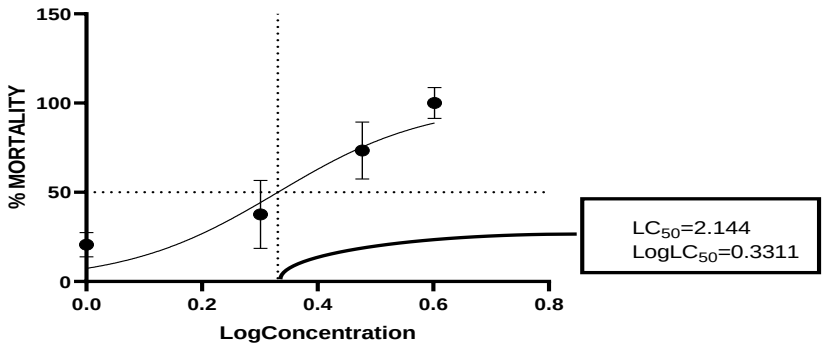

Fig. 1. Percentage mortality vs log concentration of Dolutegravir in *Drosophila melanogaster*

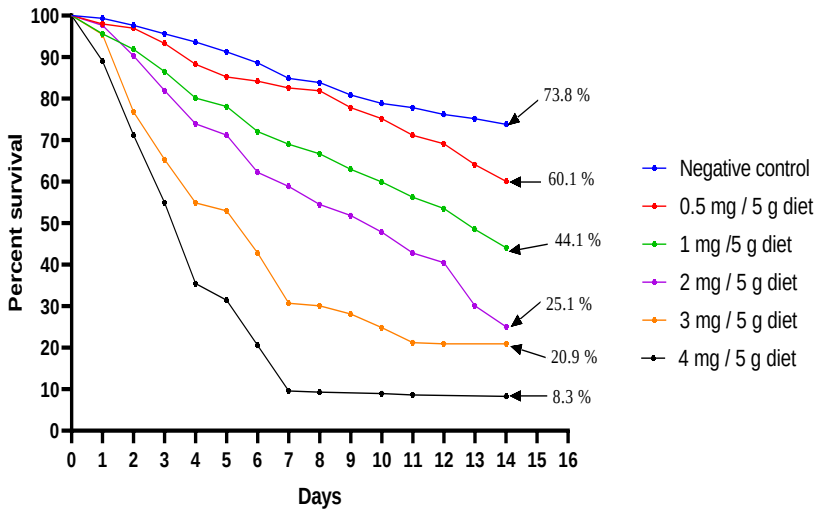

Fig. 2. 14-day survival assay for Dolutegravir showing percentage survival vs days

3.2.2 Fourteen-day survival assay for extract

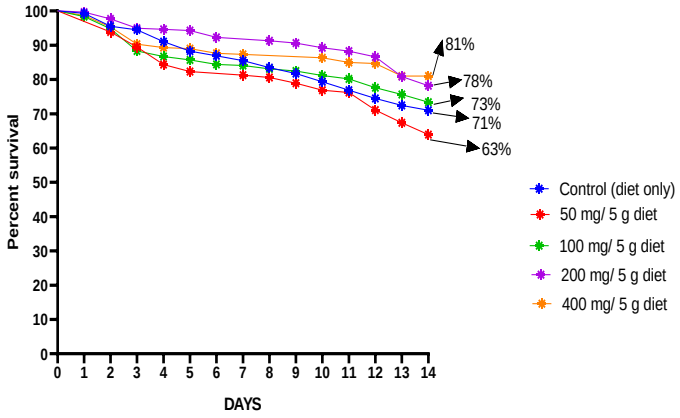

Fig. 3. 14-day Survival assay to different concentrations of the extract

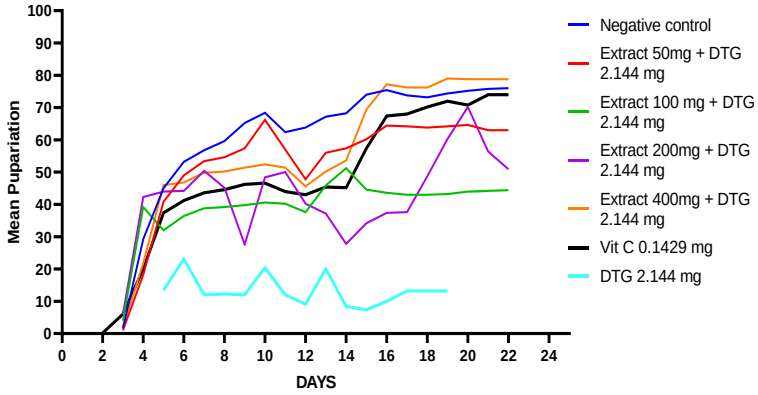

Fig. 4. Mean pupariation vs days in flies treated with the extract and DTG

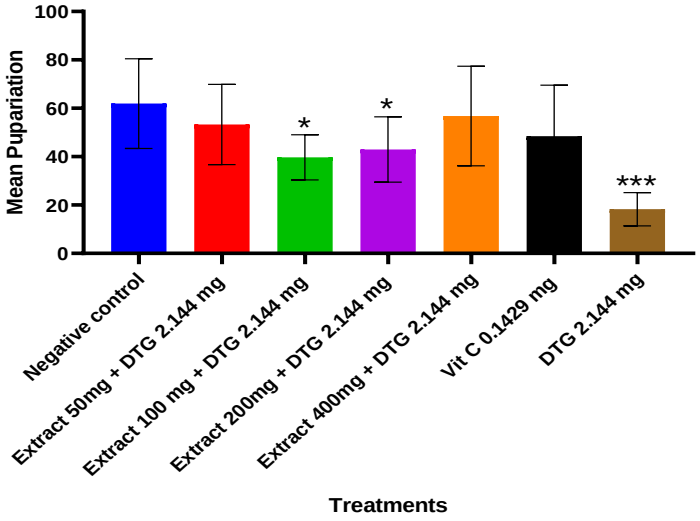

Fig. 5. Effect of treatment on mean number of pupae emerged

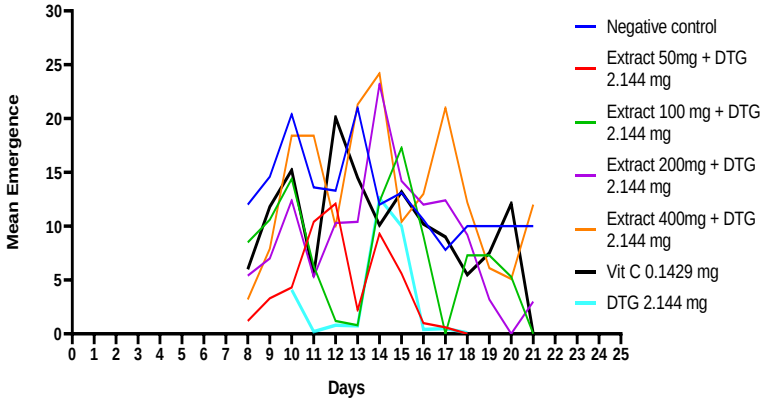

Fig. 6. Effect of treatment on cumulative number of pupae emerged

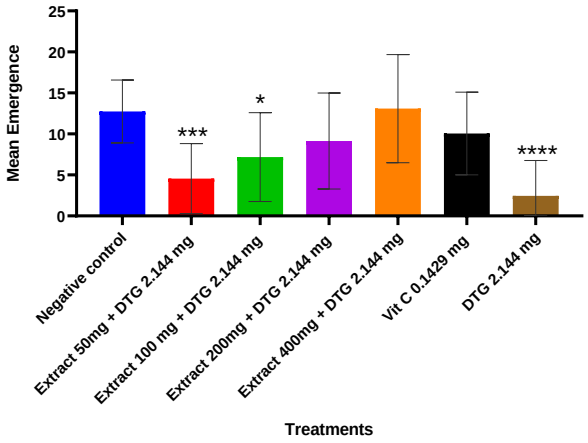

Fig. 7. Effect of treatment on mean emergence

3.3 Reproductive Ability

There was a delay in pupariation and decrease in mean pupariation in *D. melanogaster* exposed to DTG alone while there was an improvement in same parameters in those pre-treated with the extract before exposure to Dolutegravir. D. melanogaster pretreated with up to 200 and 400 mg extract showed emergence that was comparable to those in the negative and positive groups. A significant decrease was seen in the groups exposed to 50 and 100 mg extract suggesting that there was no protection at these doses.

3.3.1 Pupariation

There was a significant difference ($p<0.05$) in Pupariation for groups treated with DTG only, 100 and 200 mg of the Extract.

3.3.2 Emergence

Day-wise emergence pattern of the adult *D. melanogaster* treated with different concentrations of the extract and thereafter with dolutegravir (2.144 mg) showed considerable variations in the emergence. *D. melanogaster* that received 200 and 400 mg per 5 g diet had mean emergence even higher than those in the control group and even in the group treated with vitamin C, a known antioxidant. The emergence time for D. melanogaster in the control groups, and groups pretreated with the extract began on day 8 continued up to day 21 except for the group pretreated with the lowest dose of the extract (50 mg per 5 g diet) for which emergence stopped at 18 days however for groups treated with DTG only there was a delay in emergence (beginning at day 10) and it seized by day 17.

There was a significant difference in the groups treated with DTG only, 50 and 100 mg of the Extract.

4. DISCUSSION

The phytochemicals present in *B. oleracea* aqueous leaf extract included Alkaloids, Saponins, Tannins, Flavonoids and Carbohydrates. A large number of natural compounds present in food materials have been reported to possess antioxidant properties [18]. Despite the prevalence of antioxidants such as vitamin C and E, the majority of the antioxidant activity of fruits, vegetables, spices, and herbs may be from compounds such as phenolic acids and flavonoids considered to be much greater than that of the essential vitamins [19,20]. The presence of such phytochemicals in the *B. oleracea*, therefore, suggests that it has antioxidant properties. Comparisons between the fully sequenced *D. melanogaster* and human genomes revealed that approximately 75 % of known human disease genes have a recognizable match in the genome of *D. melanogaster* consolidating its legitimacy as a model organism for medical research (Reiter et al. 2001*)* hence the choice of the D. melanogaster as a model.

Toxicity assay carried out showed significant toxicity in the *D. melanogaster* with LC_{50} being 2.144mg per 5 g diet. As reported by Loomis & Hayes, 1996, in *Classification of LD_{50} based on dose range*, substances with LD_{50} below 5 mg/ kg are classified to be extremely toxic while substances with LD_{50} above 15,000 mg/kg are termed relatively harmless [21]. This indicates the drug DTG is moderately toxic (50-500 mg/kg) to *D. melanogaster* at the doses used.

From the 14-day survival assay of *D. melanogaster* treated with the extract, the higher percentage of survival of the flies treated with extract at 100, 150, 200 and 400 mg is indicative of the extracts ability to prolong life in *D. melanogaster* and implies its antioxidant effects. The decrease in survival of flies treated with DTG when compared to the control is indicative of toxicity of the drug on the fruit fly *D. melanogaster*. With reasonably sized longevity experiments, differences as low as 1-2% are often highly statistically significant, but the overall impact of the intervention on health status may be minor. Therefore, both statistical and biological significance must be considered when interpreting the overall results of the experiment. Inference about the aging process from survival experiments can be augmented by measures of age-related declines in behavioral or physiological health measures, including climbing ability [22] and gastrointestinal wall integrity [23]. This, therefore, suggests significant differences in survival rate when exposed to different concentrations of the drug, the extract showed an ability to significantly improve survival and this can be attributable to the presence of alkaloids, flavonoids, and other phytochemicals that have been reported to have antioxidant activity.

The delay in pupariation (day 5) and decrease in mean pupariation seen with D. melanogaster exposed to dolutegravir without pre-treatment with the extract and other groups is indicative of toxicity while the increase in pupariation in groups pretreated with 100 and 200 mg of the Extract is indicative of the protective effects of the extract. In insects, localized tissue injury often leads to global (organism-wide) delays in development and retarded metamorphosis. In Drosophila, for example, injuries to the larval imaginal discs can retard pupariation and prolong metamorphosis. Injuries induced by treatments such as radiation, mechanical damage, and induction of localized cell death can trigger similar delays. In most cases, the duration of the developmental delay appears to be correlated with the extent of damage, but the effect is also sensitive to the developmental stage of the treated animal. The proximate cause of the delays is likely disruption of the ecdysone signaling pathway, but the intermediate steps leading from tissue injury and/or regeneration to that disruption remain unknown [24]. In insects, various types of tissue damage can trigger a systemic injury response which results in prolonged larval and/or pupal stages [25].

The mean emergence in D. melanogaster pretreated with the extract was higher than those in the control group and also higher than the group treated with vitamin C, a known antioxidant. This is an evidence of the extract's ability to protect against DTG-induced toxicity and negative effects on fly emergence [26]. The longer emergence time seen in D. melanogaster pretreated with the extract and in the control groups as compared to groups exposed to only DTG also shows protection. In a previous study, evaluation of reproductive capacity in DTG-HAART exposed *D. melanogaster* showed significant ($P<0.001$) reduction in fly emergence at lower concentrations with 100 % emergence failure at higher experimental concentrations.

The exposure of DTG-HAART naïve D. melanogaster showed a significant (P<0.001) reduction in emergence without eclosion failure. This observation implies that DTG-HAART may have altered more overwhelmingly the reproductive capacity in the exposed adult flies than the developmental toxicity at the eclosion stage [27].

5. CONCLUSION

The extract of *B. oleracea* was found to possess antioxidant activity. The extract showed protective roles on dolutegravir-induced changes in pupariation and emergence of *D. melanogaster*. This study concludes that *B. oleracea* leaf extract, at certain concentrations, is able to protect against Dolutegravir-induced changes in pupariation and emergence in *D. melanogaster.*

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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