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Original paper

Some preliminary results regarding the effects of grape pomace on memory and anxiety in mice

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Abstract Lately there is an increased interest in understanding the biotechnological and possible treatment-related relevance of various natural extracts products in most of the current pathologies. Grape pomace extract was previously cited for its beneficial effects in some metabolic disorders, including diabetes. There is also a recent interest regarding the effects of grape pomace in the superior cognitive functions, however with some controversial results in this area of research. In the present paper we want to see the effects of 300 mg/kg grape pomace extract, 3 days per os administration, on the immediate spatial memory and possible locomotor activity (spontaneous alternations and number of arm entries, as tested in Y maze task), as well as on anxiety behaviour (time/number of arm entries in the open/closed arms of the maze, head drippings in the open arms, stretching in the closed arms and grooming behaviour) as tested in the elevated plus maze task in mice. In this report we present for the first time in our best of knowledge some anxiolityc effects of grape pomace, as demonstrated by the behavioral modifications in the elevated plus maze task (e.g. significant increase in the number of open arms entries), as well as some possible immediate (spatial) memory deficits as determined through the spontaneous alternations percentage in the Y maze task.

Keywords Grape pomace, memory, anxiety, mice.

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Introduction

Lately there is an increased interest in understanding the biotechnological and possible treatment-related relevance of various natural extracts products in most of the current pathologies (KINGSTON [1], DIAS et al [2]), including of course the neuropsychiatric disorders such as Alzheimer's disease, Parkinson's disease, affective disorders or schizophrenia, as our research group also previously demonstrated (FOYET et al [3], HRITCU et al [4], FOYET et al [5], GUENNÉ et al [6], BALMUS et al [7], FOYET et al [8], ANTIOCH et al [9]).

Thus, regarding the grape pomace, which is a well-known recyclable resource resulting from winemaking processes (TSUKADA et al [10], PARRY et al [11]), there is also an increased interest in its biotechnological value, with 3 detailed reports about this matter in Plos One journal alone (one about grape seeds extract) in the last few couple of years (TSUKADA et al [10], SANHUEZA et al [12], CHEAH et al [13]), related to its antibiotic-related effect on Staphylococcus aureus and *Escherichia coli* (SANHUEZA et al [12]), oxidative stress-related effects (we will insist immediately on this matter) (TSUKADA et al [10]), or the effects of grape seeds extract on a rat model of mucositis and its chemotherapeutic manifestations in colon cancer cells (CHEAH et al [13]).

In addition, grape pomace extract was previously cited for its beneficial effects in some metabolic disorders, including diabetes, such as in the study of RODRIGUEZ et al [14] which demonstrated that simple grape pomace, as well as grape pomace extract facilitated insulin signalling in a rat model of metabolic syndrome based on high-fat-fructose diet or the grape pomace induced protective effects against oxidative-stress and inflammation in a mice model of obesity (HOGAN et al [15]), as well as the studies of Li group in Virginia University (JAMSHIDZADEH et al [16]), which showed that grape pomace aqueous extract could actually protect against developing high-fat induced diabetes and is reducing inflammation (LI et al [11]).

There is also a recent interest regarding the effects of grape pomace in the superior cognitive functions, however with some controversial results in this area of research, since some groups (JAMSHIDZADEH et al [16]) showed for example that 3 different dosages of grape pomace (25, 50 and 100) or grape seed extract did not resulted in any significant modifications in spatial memory, as tested in the Morris water maze task (JAMSHIDZADEH et al [16]), while the SARKAKI et al group [17] reported clear improvements in passive avoidance task on a rat model of ischemia as a result of grape seed extract administration. Also, we should mention that to our best of knowledge, there is no previous study regarding the administration of grape pomace on anxiety processes in rodent models.

As mentioned above, one of the possible mechanisms explaining these protective effects of grape pomace in the aforementioned disorders could be related to the oxidative stress status modifications, considering that recent reports in this area of research showed an antioxidant potential from grape pomace (HOGAN et al [15], GOUTZOURELAS et al [18], AL HAKEEM [19]), as this could be relevant in the context of oxidative stress implications in most of the neuropsychiatric disorders, as we showed previously (CIOBICA et al [20], [21],[22], PANTEA STOIAN et al [23], NICOLAE et al [24], TROFIN et al [25], BALMUS et al [26]. In fact, our group is also currently working on determining the relevance of grape pomace extract on oxidative stress status, in the context mentioned above.

Thus, considering the aforementioned aspects, in the present paper we want to see the effects of 300 mg/kg grape pomace extract, 3 days per os administration, on the immediate spatial memory and possible locomotor activity (spontaneous alternations and number of arm entries, as tested in Y maze task), as well as on anxiety behaviour (time/number of arm entries in the open/closed arms of the maze, head drippings in the open arms, stretching in the closed arms and grooming behaviour) as tested in the elevated plus maze task in mice.

Materials and Methods

1. Animals

The subjects (n=8) were experimentally naive mice, weighing approximately 25-40 g at the beginning of the experiment. The animals were housed in a temperature- and light-controlled room (22°C, a 12-h cycle starting at 08:00 h) and were feed and allowed to drink water ad libitum. Mice were treated in accordance with the guidelines of animal bioethics from the Romania and all procedures were in compliance with the European Communities Council Directives.

Local Ethics Committee were informed and approved the study and also, efforts were made to minimize animal suffering and to reduce the number of animals used.

2. Treatment

Grape pomace, is the residue obtained from the grapes processing in the wine industry and will be referred to as the TES extract. After 7 days of fermentation, the plant material was dehydrated in a vacuum oven at 40°C and then ground. TES extract was administrated orally in a dosage of 300 mg/kg, 3 days in a row (solution concentration was 15mg/mL- for example 0,8mL grape pomace for a mice weighting 40 grams). Controls received per os carboxy methyl cellulose sodium (CMC) Na in a dosage of 80 mg/kg. In the immediate next days (day 4 and 5) Y maze (day 4) and elevated plus maze (day 5) tasks were performed, as described in the next section.

3. Y-maze task

Short-term memory was assessed by spontaneous alternation behaviour in the Y maze task. The Y-maze used in the present study consisted of three arms and an equilateral triangular central area. The mice was placed at the end of one arm and allowed to move freely through the maze for 8 minutes. An arm entry was counted when

the hind paws of the rat were completely within the arm. Spontaneous alternation behaviour was defined as entry into all three arms on consecutive choices. The number of maximum spontaneous alternation behaviours was calculated as the total number of arms entered minus 2 and percent spontaneous alternation was calculated as (actual alternations/maximum alternations) X 100. Spontaneous alternation behaviour is considered to reflect spatial working memory, which is a form of short-term memory (GURZU et al [27]).

4. Elevated plus maze

The elevated plus maze we used in this experiment consisted of four arms, elevated off the ground. Two arms were enclosed by walls and the other two arms were exposed. The experiment began by transferring the rats to the test room, where they were allowed to rest for one hour. Each rat was gently placed in the central area with its nose facing one of the closed arms and allowed to freely explore the maze for 5 min. Each animal was used just once. The time spent in each arm type was recorded, as well as the entries into either open or closed arms. An arm entry was counted when all four limbs of the rat were within an arm. Also, some specific behavioural parameters such as head dipping in open-arms (sticking the head below the level of the maze and towards the floor), protected stretch-attend postures (stretching) in the closed arms (the animal stretches with the forepaws while maintaining the hind paws in the same place and then retracts to the original position) and grooming (cleaning of any part of the body with the paws and/or the mouth) were measured (CRUZ et al [28]).

The percentages of time spent in the open arms (time spent in the open arms/time spent in all arms X100) and also the percentage frequency of entries in open arms (frequency of entries into open arms/total entries into all arms X 100) were then calculated. This test is based on the natural aversion of rodents for open spaces. In this way, both items described above (percentages of time spent into the open arms/frequency of entries into open arms) are considered to reflect fear-induced inhibition from entering the open arms and can be related to the anxiety level experienced by the rat (RODGERS et al [29]). Also, the measurement of the other behavioural parameters increases the sensitivity of the elevated plus maze as an experimental model of anxiety (ESPEJO et al [30]). However, their relevance will be detailed in the discussion section. Additionally, the number of entries into closed arms is believed to reflect locomotor activity (BILD et al [31]).

5. Data analysis

The animal's behaviour in Y-maze and elevated plus maze was statistically analyzed by using Student's t-test (two tailed, unpaired). All results are expressed as mean ± SEM. $p < 0.05$ was regarded as statistically significant.

Results

Regarding the first behavioural task we used, the Y maze task, we could observe a significant decrease in the spontaneous alternation percentage in the grape pomace group, when compared to controls (p=0.002) (Figure 1).

Also, the other behavioral parameter determined here showed no significant modifications between grape pomace group and controls (p=0.87) (Figure 2), suggesting that the aforementioned modifications in the spontaneous alternation behaviour was not due to some motor modifications (BILD et al [32]).

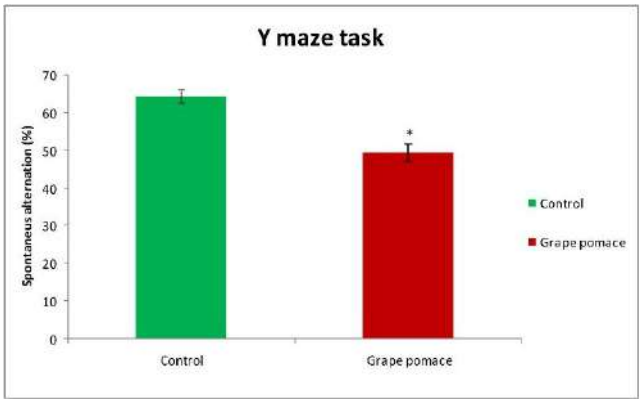

Figure 1. Effects of grape pomace administration (3 days, 300 mg/kg) on spontaneous alternation behaviour evaluated in the Y-maze test. The values are mean ± SEM (n=5 animals per control and n=3 for grape pomace group). *p = 0.002 vs. control group.

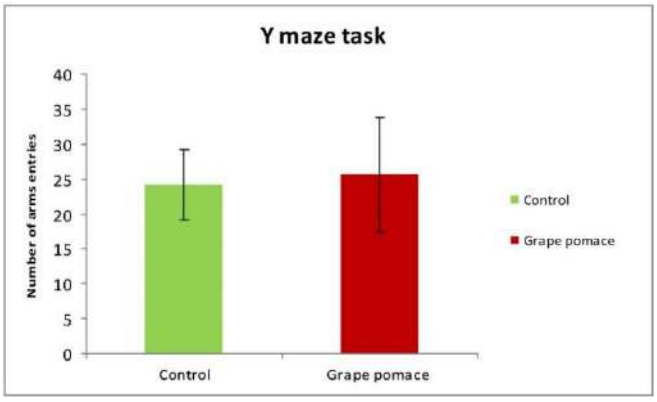

Figure 2. Effects of grape pomace administration (3 days, 300 mg/kg) on number of arm entries in the Y-maze test. The values are mean ± SEM (n=5 animals per control and n=3 for grape pomace group).

Regarding the second behavioral task we used, that was represented by elevated plus maze, which is a test showing mainly the anxiety-related levels in rodents (CIOBICA et al [33]), we could observe some anxiolytic effects for the grape pomace group vs. the controls as parameters such as number of arms entries in the open arms were significantly increased in the extract group, as compared to controls (p=0.047) (Figure 3).

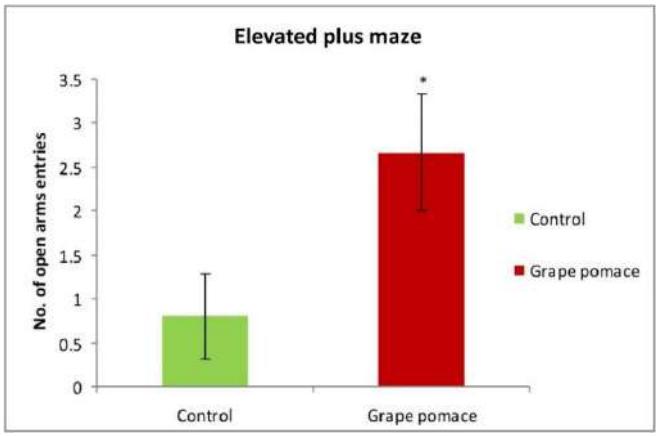

Figure 3. Effects of grape pomace administration (3 days, 300 mg/kg) on number of arms entries in the open arms evaluated in the elevated plus maze task. The values are mean ± SEM (n=5 animals per control and n=3 for grape pomace group). *p = 0.047 vs. control group.

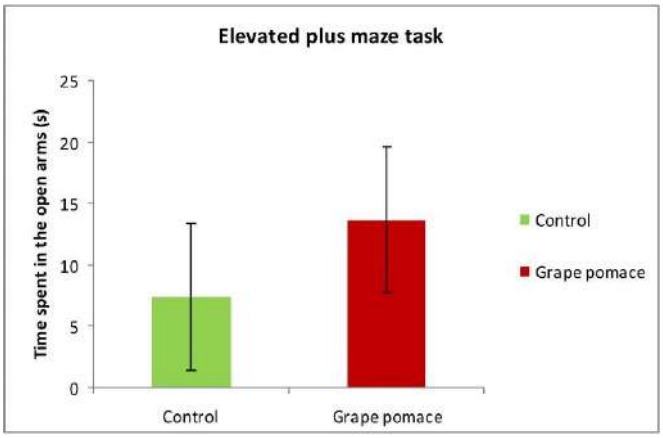

Figure 4. Effects of grape pomace administration (3 days, 300 mg/kg) on time spent (s) in the open arms evaluated in the elevated plus maze task. The values are mean ± SEM (n=5 animals per control and n=3 for grape pomace group).

These anxyolitic effects are also suggested by an increased time spent in the open arms of the elevated plus maze in the pomace group vs. controls (Figure 4), increased number of head dipping in the open arms (e.g. sticking the head below the level of the maze and towards the floor), in the extract group as compared to controls (Figure 5) and a decreased number of grooming behaviours vs. controls in pomace group vs. control mice (Figure 6), although in all of these 3 cases the modifications were not statistically significant, probably considering the reduced number of animals which we used.

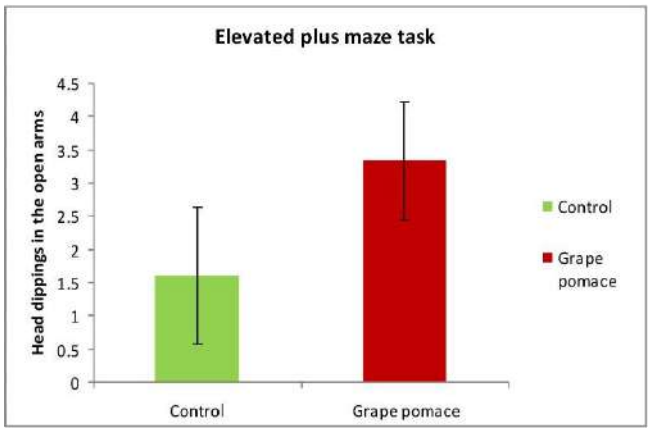

Figure 5. Effects of grape pomace administration (3 days, 300 mg/kg) on number of head dippings in the open arms evaluated of elevated plus maze task. The values are mean ± SEM (n=5 animals per control and n=3 for grape pomace group).

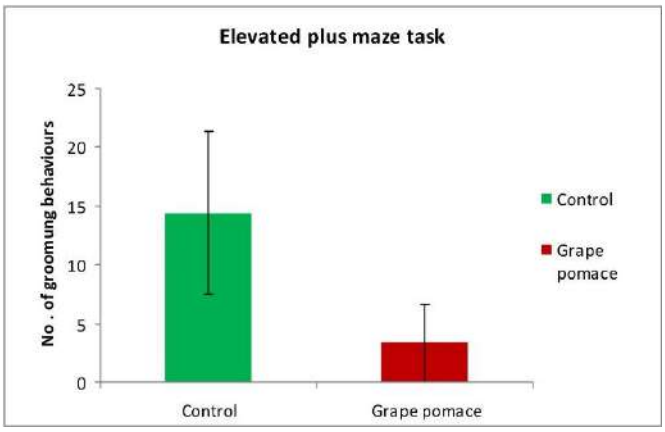

Figure 6. Effects of grape pomace administration (3 days, 300 mg/kg) on grooming behaviour evaluated in the elevated plus maze task. The values are mean ± SEM (n=5 animals per control and n=3 for grape pomace group).

On the other side, the number of stretching behaviours in the closed arms (the animal stretches with the forepaws while maintaining the hind paws in the same place and then retracts to the original position – a behaviour suggesting anxiogenic manifestations – (BILD et al [31]) was not modified between the 2 groups (Figure 7).

We should also mention here that the number of closed arms entries, which is considered a parameter suggesting the locomotor activities of mice in the elevated plus maze, was only slightly increased (but not statistically significant, p=0.081) in the mice which received grape pomace, when compared to controls (Figure 8).

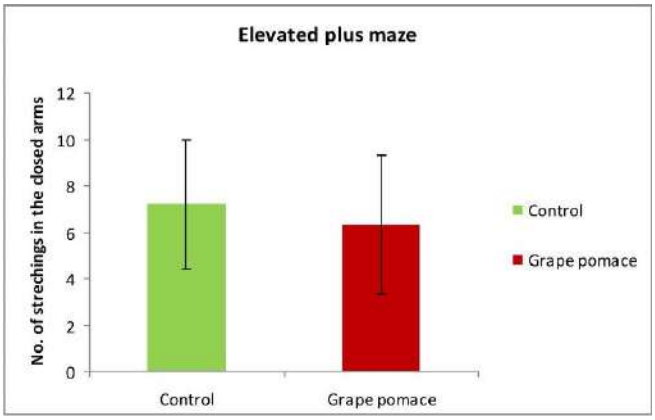

Figure 7. Effects of grape pomace administration (3 days, 300 mg/kg) on number of strechings in the closed arms evaluated in the elevated plus maze task. The values are mean ± SEM (n=5 animals per control and n=3 for grape pomace group).

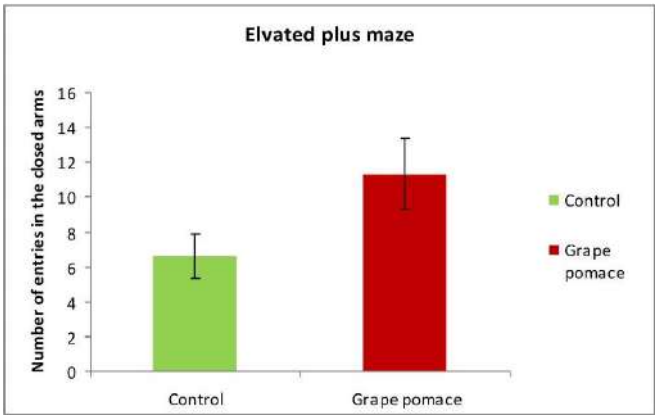

Figure 8. Effects of grape pomace administration (3 days, 300 mg/kg) on number of arms entries in the closed arms of elevated plus maze task. The values are mean ± SEM (n=5 animals per control and n=3 for grape pomace group).

Discussion

In this report we present for the first time in our best of knowledge some anxiolityc effects of grape pomace, as administrated orally for 3 days, in a 300 mg/kg dosage, as demonstrated by the behavioral modifications in the elevated plus maze task (e.g. significant increase in the number of open arms entries), as well as some possible immediate (spatial) memory deficits as determined through the spontaneous alternations percentage in the Y maze task.

As mentioned, previous studies regarding the cognitive effects of grape pomace showed some variable results, with the Jamshidzadeh group showing that while 100 mg of grape pomace could rescue some of the spatial memory impairments induced in the Water maze task by the administration of scopolamine in mice, but did not had any significant effect on the memory processes, when administrated alone in various dosages such as 25, 50 or even 100 mg/kg (JAMSHIDZADEH et al [16]), while on the other side some other reports demonstrated that grape pomace could facilitate the molecular processes of learning such as long term potentiation and memory long-term retention in a behavioral paradigms such as passive avoidance task (SARKAKI et al [17]).

These different results could be explained by the different dosages of grape pomace used, the different routes of administration, different behavioral tasks implicated or different species of animals used for the experiments (e.g. rat vs. mice).

Also if we analyze the results of grape pomace administration on elevated plus maze task, we could see some possible anxyolitic effects, which are reported here by our group for the first time in our best of knowledge, as demonstrated by a significant increase in the number of open arms entries, as well as by an increased number of head dippings in the open arms (e.g. sticking the head below the level of the maze and towards the floor), an increased time spent in the open arms of the elevated plus maze and a decreased number of grooming behaviours in the grape pomace mice vs. the controls. In this way, it can be speculated that Y–maze deficiencies could be correlated to a decreased anxiety-related behaviour and to an increased exploratory activity, although it should be mentioned that the number of closed arm entries in the elevated plus maze task was not statistically different between our research groups.

Considering the mechanistics behind some of these positive effects of grape pomace (as it was suggested for some time that moderate wine intake could exert some protective effect on neurological deficiencies – (LETENNEUR et al [34]), as we previously mentioned, some antioxidant effects are cited by some authors, which showed for example that grape pomace could actually increase glutathione, gamma-glutamylcysteine synthetase levels or glutathione S-transferase activity at the muscular and endothelial cells level (GOUTZOURELAS et al [18]). Also, grape pomace is decreasing lipid peroxidation when used as a biotechnological adjuvant in some food industry applications (AL HAKEEM [19]).

However, there are controversies in this area of research also, with reports demonstrating that grape pomace could not improve the increased oxidative stress (as determined by the levels of GPX or lipid peroxidation markers) status associated with a mice model of obesity (HOGAN et al [15]) .

In addition, there are also studies such as the one conducted by the Tsukada group in 2016 and published in Plos One journal, which showed that photo irradiated grape pomace extract could exert some prooxidative

effects, with possible applications as a bactericidal compound (TSUKADA et al [10]).

In the same context, we could mention that grape pomace extract could exert some anti-inflammatory, considering that for example the Li group showed that grape pomace could actually down regulate some genes and cytokines associated with the inflammatory processes in a streptozocin-induced rat model of diabetes (LI et al [11]), while other demonstrated decreased protein-C-reactive levels, as a result of grape pomace administration, in the plasma of experimental obese mice (HOGAN et al [15]).

In fact, these antioxidant and anti-inflammatory effects of grape pomace could also explain the beneficial effects of this compound in different models of diabetes (LI et al [11], RODRIGUEZ et al [14], HOGAN et al [15]), considering that oxidative stress is implicated in the mechanistics of various metabolic disorders (TIMOFTE et al [35]), together with a variety of other additional risk factors (SERBAN et al [36], TOARBA et al [37], VLASCEANU et al [38], CRACIUN et al [39]).

In fact, our research team is working now in better understanding the relevance of grape pomace orally administration on the oxidative stress modifications in various organs of mice.

In addition, we could mention here that similar protective aspects were found also for grape seed extract, with antioxidant effects on metabolic-related disorders (CHIS et al [40], EL-AWDAN et al [41]) or in other specific diseases such as mucositis or colon cancer (CHEAH et al [13]).

Thus, as this a rather new area of research, future studies should better establish the effects of this compound in most of the cognitive and complex pathological functions, with a more diverse experimental approach of dosages used and a more complex battery of behavioural tests implicated, as our group is already planning for future designed studies.

In fact, regarding the limitations of our study we can mention here the small numenr of animals used, due to the ethical regulations, as well as the fact that the extarct was administrated only for 3 days, as this was limited by the fact that 3 mice for the grape pomace group passed away in the third day of treatment, probably due to overdosing. This aspects lead to an urgent stop of the treatment and the beginning of the behavioral tasks. In this way, future studies with smaller dosages are underway by our research group. Also, considering the possible relevance of this compound in a variety of animal models of neuropsychiatric disorders developed and modified by our research group (LEFTER et al [42]) we are also working now in determining the applications of grape pomace in some models for the disorders mentioned above.

Conclusions

In this report we present for the first time in our best of knowledge some anxiolityc effects of grape pomace, as administrated orally for 3 days, in a 300 mg/kg dosage, as demonstrated by the behavioral modifications in the elevated plus maze task (e.g. significant increase in the number of open arms entries), as well as some possible immediate (spatial) memory deficits as determined through the spontaneous alternations percentage in the Y maze task. Future studies implying different dosages, duration of treatment and possible usage in some animal models of neuropsychiatric seems warranted and are underway by our research group.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

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