Evaluation of Acute and Chronic Effects of D-Galactose on Memory and Learning in Wistar Rats

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Objective: D-galactose has been commonly used in rodent models to induce accelerated effects of aging, including those on learning, memory, and muscular tone and coordination. This is normally seen on chronic administration of D-galactose. However, there is minimal suggestive evidence on the short-term effects of the same. The aim of the study was to study the acute and chronic effects of D-galactose on learning and memory in Wistar rats.
Methods: Twenty four male Wistar rats were randomly assigned to the control, standard (rivastigmine), oral D-galactose (200 mg/kg/day) and subcutaneous D-galactose (200 mg/kg/day) for a total duration of 8 weeks. Effects on learning and memory were assessed at 2 weeks, 4 weeks and 8 weeks by Morris water maze model and passive avoidance testing.
Results: Both oral and subcutaneous D-galactose showed positive effects on learning and memory on acute dosing, whereas this beneficial effect was lost during chronic dosing.
Conclusion: Short-term administration of D-galactose showed positive effects, while long-term administration nullified these effects.

KEY WORDS: Morris water maze; Passive avoidance; Rivastigmine; Alzheimer's disease.

INTRODUCTION

D-galactose (D-gal) is a physiological nutrient that is chemically a reducing sugar.[1] It is found in abundance in milk, vegetables and fruits.[2] D-gal interacts with various free amines in the protein architecture via non-enzymatic glycation, resulting in the generation of advanced glycation products. This results in the generation of reactive oxygen species (ROS). Also, excessive levels of D-gal in the body (either due to increased intake or decreased metabolism) may lead to the formation of various ROS, chiefly through oxidative metabolism, and additionally through the secondary glycation pathway.[3] ROS and advanced glycation products have been implicated in several age-related diseases like Alzheimer's disease, diabetes mellitus, cancers and amyotrophic lateral sclerosis, etc.[4]

Since D-gal induces the generation of these advanced glycation products and ROS, it is commonly employed as a model to enhance aging in rodents, thereby finding a place in rodent models for Alzheimer's disease and other memory impairment disorders. Most of the studies that are currently available in the accessible literature mention that D-gal can successfully be used as a rodent model, when used subcutaneously or intraperitoneally. However, a few studies have shown that oral administration of D-gal is ironically protective against age-related changes.[4,5]

Similarly, the temporal associations of these effects with the administration of D-gal are unclear. While most authors have mentioned that chronic administration of D-gal can lead to impairment of learning and memory in rodents, the acute effects of the sugar are not well-established. A few studies have shown that this is not a standard model, as these changes produced are not consistent across all species and ages.[6]

Rivastigmine, a cholinesterase inhibitor has a comparatively selective action on the enzyme in brain when

compared to skeletal muscle and heart. Its role in memory can be attributed to cholinesterase inhibition in hippocampus and cortex, thus preventing neuronal damage and enhancing cholinergic transmission, culminating in an increase in memory and retention. A study has shown that rivastigmine at a dose of 0.5-2.5 mg/kg reduced the effects of scopolamine on working memory.[7,8]

Hence, the current study was planned to evaluate the acute and chronic effects of D-gal on rodents, when administered through two different routes: subcutaneous and oral, and comparing the effects with a control group and a standard group treated with rivastigmine.

The aim of the current study was to evaluate the acute and chronic effects of D-gal on memory and learning in Wistar rats using two models: Morris water maze and passive avoidance test, at 2 weeks, 4 weeks and 8 weeks.

METHODS

Animal Selection and Handling

Twenty four male Wistar rats, weighing between 150 and 200 g, were acquired from the Central Animal Research Facility, Manipal University. The rats were randomly assigned to one of the four groups, and their tails were marked with different colours for identification purposes. A maximum of 4 rats were housed in a single polypropylene cage, to avoid overcrowding. All rats were given standard laboratory feed and water *ad libitum*.

Ethical Clearance

Clearance was obtained from the Institutional Animal Ethics Committee (IAEC number: IAEC/KMC/35/2015), and the experiment was conducted in accordance with the CPCSEA guidelines.

Drugs

Rivastigmine was used as standard therapy (dose was selected based on 2 previous studies),[8,9] while D-gal was used in the test groups. Normal saline was used in the control group. All drugs were administered once daily for a total duration of 8 weeks.

Experimental Groups

The different experimental groups were as follows (with 6 rats in each group): Group 1 (Control), normal saline (orally); Group 2 (Test group 1), D-gal 200 mg/kg/day (orally); Group 3 (Test group 2), D-gal 200 mg/kg/day (subcutaneously); Group 4 (Standard), rivastigmine 5 mg/kg/day (orally).

Test Procedures

Two models were used to assess the effect of the test and standard drugs on learning and memory—Morris water maze test and passive avoidance test.

Morris water maze[5,10]

All rats were accustomed to the water maze for a duration of 2 days before the training period, wherein they were allowed to freely swim. On the first day of acclimatization, no quadrants were marked, whereas on day 2, they were marked. Following this, the rats were given a training to identify a hidden platform (submerged about 2 cm below the water level in the north-west quadrant), in a pattern of 4 entries into the maze (1 from each quadrant) at an inter-entry interval of 1 minute each. The same was repeated thrice in a day for 4 days, separated by time intervals of 30 minutes each.

Following this training period, a baseline assessment was done, during which the swimming distance (the total distance that a rat travels before it reaches the hidden platform), swimming speed, north-west latency (the time taken by the rats to reach the north-west quadrant) and escape latency (the time taken for the rats to reach the hidden platform) were noted down from the automated software. The rats were then given the respective drugs, and again tested at 2 weeks, 4 weeks and 8 weeks. Training was done only once at the beginning of the experiment, as per protocol.

Passive avoidance test[5,11]

A step-through model of passive avoidance testing was followed in the study. A two-chambered box with a brightly-lit compartment and a darker (with a base that delivers a small electric shock) "punishing" compartment was utilized. On day 1, the rats were acclimatized to the new environment. They were allowed to freely roam in the box. On day 2, a training session was conducted, wherein the rats were allowed to learn that the brightly-lit chamber is the safer option for them to stay in. On day 3, the actual test was conducted without the electric charge, to assess how many of the animals remembered that they had to stay in the brightly-lit compartment. Escape latency

(the time taken for the rats to reach the dark chamber) and retention time (the total time spent by the rats in the brightly-lit chamber) were noted down manually at baseline, 2 weeks, 4 weeks and 8 weeks.

Euthanasia

After the experiment was concluded, all animals were humanely sacrificed by administering an overdose of ketamine, and then incinerated.

All values were tabulated and analyzed using IBM SPSS ver. 22.0 (IBM Co., Armonk, NY, USA). Mean± standard error of mean was used to express each parameter. Comparisons among the various experimental groups was performed using one-way analysis of variance (ANOVA), followed by *post-hoc* Tukey's test. The *p* value of less than 0.05 was considered as statistically significant.

RESULTS

Morris Water Maze

Swimming distance

Swimming distance was captured from the automated software, based on the total distance that a rat travels before it reaches the hidden platform. Animals with im-

paired memory and learning are bound to travel longer before they can arrive at the set platform.

As shown in Table 1 and Figure 1, the baseline distances were all comparable with each other, and no statistical differences were found ($p>0.05$). At 2 weeks, the swimming distance was found to be significantly reduced in groups 2 ($p=0.018$) and 4 ($p=0.005$), when compared
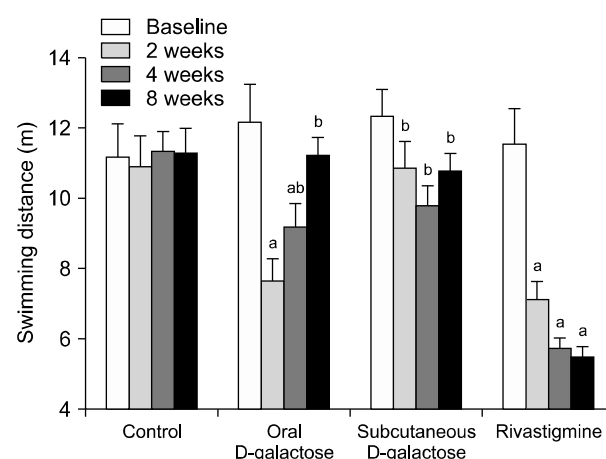

Fig. 1. Swimming distance (m) assessed by Morris water maze test at different time points.
a denotes $p<0.05$ versus control group; b denotes $p<0.05$ versus standard group; p value calculated using one-way ANOVA followed by Tukey's *post-hoc* test.

Table 1. Swimming distance assessed by Morris water maze test at different time points

Group		Swimming distance (m)			
		Baseline	2 weeks	4 weeks	8 weeks
1	Control	11.17±0.93	10.90±0.87	11.33±0.56	11.29±0.70
2	Oral D-galactose	12.16±1.07	7.65±0.63[a]	9.19±0.65[ab]	11.23±0.49[b]
3	Subcutaneous D-galactose	12.33±0.76	10.86±0.75[b]	9.79±0.56[b]	10.77±0.50[b]
4	Rivastigmine	11.54±0.99	7.13±0.50[a]	5.73±0.29[a]	5.50±0.29[a]

Values are presented as mean±standard error of mean.
[a]$p<0.05$ versus control group; [b]$p<0.05$ versus standard group; p value calculated using one-way ANOVA followed by Tukey's *post-hoc* test.

Table 2. Swimming speed assessed by Morris water maze test at different time points

Group		Swimming speed (m/s)			
		Baseline	2 weeks	4 weeks	8 weeks
1	Control	0.10±0.01	0.11±0.01	0.11±0.01	0.11±0.00
2	Oral D-galactose	0.10±0.00	0.17±0.01[a]	0.19±0.01[ab]	0.12±0.01[b]
3	Subcutaneous D-galactose	0.11±0.00	0.14±0.00[ab]	0.15±0.01[ab]	0.11±0.01[b]
4	Rivastigmine	0.11±0.01	0.19±0.01[a]	0.23±0.01[a]	0.30±0.01[a]

Values are presented as mean±standard error of mean.
[a]$p<0.05$ versus control group; [b]$p<0.05$ versus standard group; p value calculated using one-way ANOVA followed by Tukey's *post-hoc* test.

to control. At 4 weeks, the distances travelled by rats in groups 2 (p=0.045) and 4 ($p<0.001$) were again significantly reduced when compared to that of the control group. At 8 weeks, only group 4 ($p<0.001$) showed statistically significant reduction in swimming distance when compared against control.

Swimming speed

Swimming speeds were captured from the automated software till the rats reached the hidden platform. Animals with impaired memory and learning are bound to travel slower.

As shown in Table 2 and Figure 2, the baseline speeds were all comparable with each other, and no statistical differences were found ($p>0.05$). At 2 weeks, the swimming speed was found to be significantly increased in groups 2 ($p<0.001$), 3 (p=0.036) and 4 ($p<0.001$), when compared to control. At 4 weeks, the speeds of the rats in groups 2 ($p<0.001$), 3 (p=0.019) and 4 ($p<0.001$) were
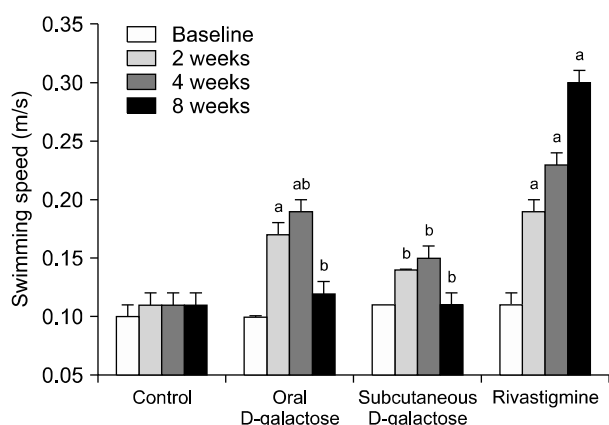
again significantly increased when compared to that of the control group. At 8 weeks, only group 4 ($p<0.001$) showed statistically significant rise in speed, when compared against control.

Escape latency

Escape latency was calculated by taking the time taken for the rats to reach the hidden platform. Animals with impaired memory and learning are bound to travel longer before they can arrive at the set platform, thereby taking more time to find the platform.

As shown in Table 3 and Figure 3, the baseline escape latency times were all comparable with each other, and no statistical differences were found ($p>0.05$). At 2 weeks, the escape latency was found to be significantly lower in groups 2 ($p<0.001$), 3 ($p<0.001$) and 4 ($p<0.001$), when compared to control. At 4 weeks, the time taken by the rats in groups 2 ($p<0.001$), 3 ($p<0.001$) and 4 ($p<0.001$) was significantly reduced when compared
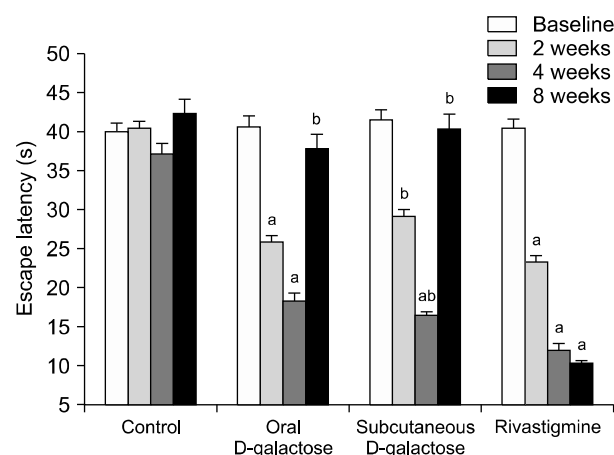

Fig. 2. Swimming speed (m/s) assessed by Morris water maze test at different time points.
a denotes $p<0.05$ versus control group; b denotes $p<0.05$ versus standard group; p value calculated using one-way ANOVA followed by Tukey's *post-hoc* test.

Fig. 3. Escape latency (s) assessed by Morris water maze test at different time points.
a denotes $p<0.05$ versus control group; b denotes $p<0.05$ versus standard group; p value calculated using one-way ANOVA followed by Tukey's *post-hoc* test.

Table 3. Escape latency assessed by Morris water maze test at different time points

Group		Escape latency (s)			
		Baseline	2 weeks	4 weeks	8 weeks
1	Control	40.00±1.06	40.5±0.76	37.17±1.17	42.33±1.38
2	Oral D-galactose	40.67±1.33	25.83±0.87[a]	18.33±1.31[ab]	37.83±0.87[b]
3	Subcutaneous D-galactose	41.50±1.26	29.17±0.79[ab]	16.50±0.43[ab]	40.33±1.28[b]
4	Rivastigmine	40.50±1.06	23.33±0.71[a]	12.00±0.86[a]	10.33±1.15[a]

Values are presented as mean±standard error of mean.
[a]$p<0.05$ versus control group; [b]$p<0.05$ versus standard group; p value calculated using one-way ANOVA followed by Tukey's *post-hoc* test.

to that of the control group. At 8 weeks, only group 4 (p <0.001) showed statistically significant fall in escape latency, when compared against control.

North-west latency

North-west latency was calculated by taking the time taken by the rats to reach the quadrant (north-west) that contained the hidden platform. Animals with impaired memory and learning are bound to take a longer time to arrive at the quadrant with the set platform.

As shown in Table 4 and Figure 4, the baseline north-west latency times were all comparable with each other, and no statistical differences were found ($p>0.05$). At 2 weeks, the north-west latency was found to be significantly lower in groups 2 ($p<0.001$), 3 ($p<0.001$) and 4 ($p=0.012$), when compared to control. At 4 weeks, the time taken by the rats in groups 2 ($p<0.001$), 3 ($p<0.001$) and 4 ($p<0.001$) was significantly reduced when compared to that of the control group. At 8 weeks, only group 4 ($p<0.001$) showed statistically significant fall in escape latency, when compared against control.

Passive Avoidance Test

Escape latency

Escape latency was calculated by taking the time taken for the rats to reach the dark chamber. Animals with impaired memory and learning are bound to travel quicker into the dark chamber.
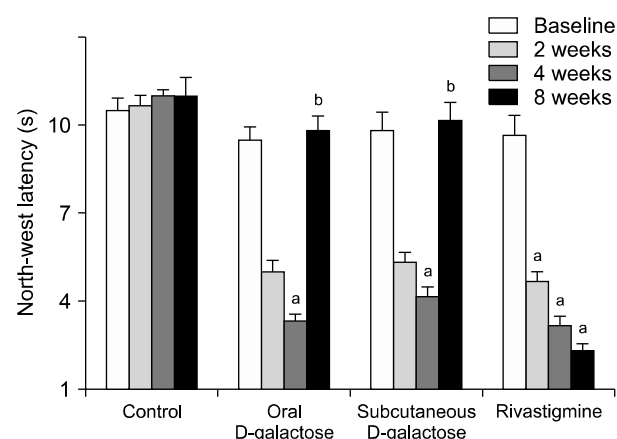

Fig. 4. North-west latency (s) assessed by Morris water maze test at different time points.
a denotes $p<0.05$ versus control group; b denotes $p<0.05$ versus standard group; p value calculated using one-way ANOVA followed by Tukey's *post-hoc* test.

Table 4. North-west latency assessed by Morris water maze test at different time points

Group		North-west latency (s)			
		Baseline	2 weeks	4 weeks	8 weeks
1	Control	10.50±0.43	10.67±0.67	11.00±0.52	11.00±0.57
2	Oral D-galactose	9.50±0.43	5.00±0.37[a]	3.33±0.21[a]	9.83±0.61[b]
3	Subcutaneous D-galactose	9.83±0.60	5.33±0.33[a]	4.17±0.31[a]	10.17±0.65[b]
4	Rivastigmine	9.67±0.67	4.67±0.33[a]	3.17±0.31[a]	2.33±0.21[a]

Values are presented as mean±standard error of mean.
[a]$p<0.05$ versus control group; [b]$p<0.05$ versus standard group; p value calculated using one-way ANOVA followed by Tukey's *post-hoc* test.

Table 5. Escape latency assessed by passive avoidance test at different time points

Group		Escape latency (s)			
		Baseline	2 weeks	4 weeks	8 weeks
1	Control	15.56±0.53	15.35±1.11	15.98±1.05	15.45±0.96
2	Oral D-galactose	15.34±0.45	39.81±2.50[a]	36.29±2.73[ab]	23.63±2.41[b]
3	Subcutaneous D-galactose	15.73±0.54	22.54±2.13[b]	22.17±2.07[b]	31.98±3.77[b]
4	Rivastigmine	15.02±0.41	37.28±4.11[a]	53.16±4.94[a]	86.32±11.38[a]

Values are presented as mean±standard error of mean.
[a]$p<0.05$ versus control group; [b]$p<0.05$ versus standard group; p value calculated using one-way ANOVA followed by Tukey's *post-hoc* test.

As shown in Table 5 and Figure 5, the baseline escape latency times were all comparable with each other, and no statistical differences were found ($p>0.05$). At 2 weeks, the escape latency was found to be significantly higher in groups 2 ($p<0.001$) and 4 ($p<0.001$), when compared to control. Also, at 4 weeks, the time taken by the rats in groups 2 ($p=0.001$) and 4 ($p<0.001$) was significantly higher when compared to that of the control group. At 8 weeks, only group 4 ($p<0.001$) showed statistically significant rise in escape latency, when compared against control.

Retention time

Retention time was calculated by taking the total time spent by the rats in the brightly-lit chamber. Animals with impaired memory and learning are bound to travel quicker into the dark chamber, and thereby have shorter retention times.

As shown in Table 6 and Figure 6, the baseline re-

tention times were all comparable with each other, and no statistical differences were found ($p>0.05$). At 2 weeks, the retention time was found to be significantly higher in groups 2 ($p<0.001$), 3 ($p<0.001$) and 4 ($p<0.001$), when compared to control. Again, at 4 weeks, the time spent in the bright chamber by the rats in groups 2 ($p=0.005$), 3 ($p=0.017$) and 4 ($p<0.001$) was significantly higher when compared to that of the control group. Similarly, at 8 weeks, only group 4 ($p<0.001$) showed statistically significant rise in retention time, when compared against control.

To summarize, our results have shown that oral D-gal 200 mg/kg/day had beneficial effects on memory and learning during the short-term administration period, whereas the same benefit was lost when the rats were dosed for longer periods. Subcutaneous D-gal 200 mg/kg/day also showed a positive trend in all parameters except for swimming distance (Morris water maze model) and escape latency (passive avoidance model), when as-
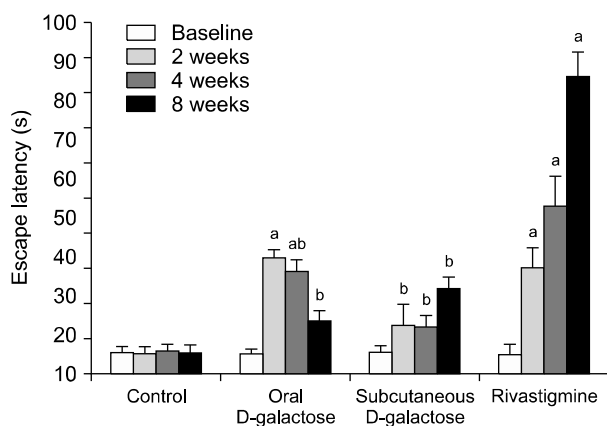

Fig. 5. Escape latency (s) assessed by passive avoidance test at different time points.
a denotes $p<0.05$ versus control group; b denotes $p<0.05$ versus standard group; p value calculated using one-way ANOVA followed by Tukey's *post-hoc* test.
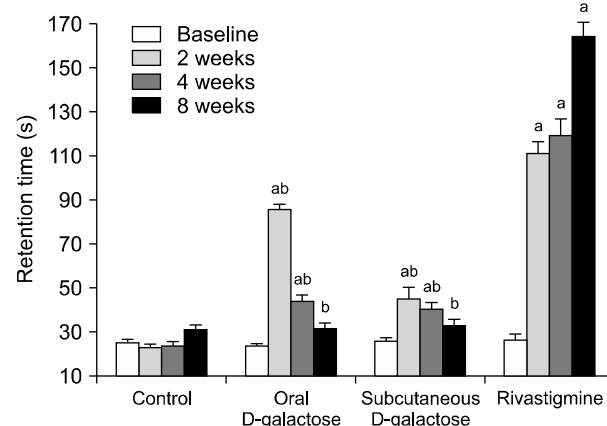

Fig. 6. Retention time (s) assessed by passive avoidance test at different time points.
a denotes $p<0.05$ versus control group; b denotes $p<0.05$ versus standard group; p value calculated using one-way ANOVA followed by Tukey's *post-hoc* test.

Table 6. Retention time assessed by passive avoidance test at different time points

	Group	Retention time (s)			
		Baseline	2 weeks	4 weeks	8 weeks
1	Control	25.13±1.46	23.05±1.56	20.67±0.88	21.99±1.98
2	Oral D-galactose	23.76±0.97	85.87±2.08[ab]	44.10±2.91[ab]	31.72±2.49[b]
3	Subcutaneous D-galactose	25.96±1.47	45.20±5.21[ab]	44.10±2.91[ab]	33.11±2.69[b]
4	Rivastigmine	26.57±2.57	111.41±5.03[a]	119.51±7.51[a]	164.62±6.06[a]

Values are presented as mean±standard error of mean.
[a]$p<0.05$ versus control group; [b]$p<0.05$ versus standard group; p value calculated using one-way ANOVA followed by Tukey's *post-hoc* test.

sessed in the acute period. Further, rivastigmine (used as the standard drug) showed beneficial effects on memory and learning in all the measured parameters, thereby reinforcing the drug as a standard comparator for models of learning and memory.

DISCUSSION

Our study revealed that acute administration of D-gal showed promising results on learning and memory at the end of 2 weeks and 4 weeks, when compared to control. However, by the end of 8 weeks (chronic administration), the results were no longer significant. In fact, they were comparable with the control or baseline values. There was no further worsening of the test results beyond the control values, probably because the duration of 2 months was not enough. Had the project been extended beyond the current duration of 2 months, worsening compared to control group might have been seen.

On careful examination of results at 2 weeks, it shows that oral D-gal given at 200 mg/kg/day showed better improvement in memory and learning when compared to 200 mg/kg/day subcutaneous D-gal. A similar study was done by Salkovic-Petrisic et al.[5] the results of which show that oral D-gal improved memory and learning when compared to parenteral D-gal, when used in streptozotocin-treated rats. However, when used alone, D-gal did not show significant results, the reason for which was not explained. Also, in this study, the effects were chiefly compared using glucose and galactose levels in the blood and CSF, and not behavioural studies.[5]

Oral administration of D-gal probably increases the levels of incretins like glucagon like peptide-1 (GLP1) and glucose dependent insulinotropic polypeptide, which promote insulin secretion.[12] GLP1 analogues have demonstrated improvement in learning and memory plus have also shown to reduce levels of hyperphosphorylated tau and total tau levels,[13] which may not be evident with parenteral routes. In our opinion, this difference might also be attributed to variations in drug bioavailability between the two routes.

However, at 8 weeks, there was deterioration in the learning ability and memory when D-gal was given through both oral and parenteral routes, compared to the results at 2 weeks and at 4 weeks, and the results were comparable with those of the control group. The mecha-

nism behind this difference at time points was not analyzed, and it could be plainly due to resistance to D-gal. The duration of the present study might have been too short to have shown any significant deterioration compared to the control group, as was seen in previous studies. In another study, D-gal induced memory loss and learning impairment on chronic use, which has been attributed to generation of free radicals, resulting in impairment of neurogenesis and ultimately, neurodegeneration.[14-16] Caspase-mediated apoptosis, which resulted in degeneration of neurons, was also seen in these studies.

A study done by Wei et al.[17] using Morris water maze model and object recognition test in 2005 using D-gal showed that mice spent less time swimming in the target quadrant and increased escape latencies. The authors had used three subcutaneous doses for a period of 8 weeks; −50, 100, and 200 mg/kg/day. D-gal impaired memory and affected cells in a dose dependent manner (50-100 mg/kg), bringing about changes of natural ageing.[17]

Another study done by Haider et al.[18] reported that short-term (1 week) high dose (300 mg/kg/day intraperitoneally) administration of D-gal showed anxiogenic and depressogenic behavior. It also altered memory functions. As mentioned in the aforementioned studies, even this was attributed to free radical-mediated damage. Superoxide dismutase activity and lipid peroxidation activity were increased significantly and the activities of glutathione peroxidase and catalase were decreased.[18] This is probably because a very high dose was used in this study as opposed to our study, wherein lower doses were used. In addition, in the present study, the routes of drug administration were different—oral and subcutaneous.

Our study showed a definite initial improvement followed by deterioration of learning and memory in both the models tested. The initial improvement could be due to a rise in the incretin levels, as mentioned before. We attribute the deterioration to free radical-mediated neuronal damage, as suggested by earlier studies.

This study has some limitations. Firstly, only one dose each of oral and subcutaneous formulations was considered in our study. So, we do not know whether the effects are dose dependent. Ideally, multiple doses should have been used. Secondly, only one animal species was utilized, and only the male gender was included. This was to remove the influence of hormonal changes in female rats. However, showing the beneficial effects of galactose on

both sexes of rats would have been ideal. Thirdly, tendency to show declined on chronic administration was observed. Ideally, the study should have been continued further to see whether the deterioration was progressive and worsening compared to control was seen. Fourthly, a high dose of rivastigmine was used in the present study, taking into account the dose used in two previous studies. And the mechanism by which D-gal improved/impaired memory and learning in the current study was not studied. Instead, the mechanisms from previous studies were considered. Lastly, neurochemical and neuro-histological assays were not performed.

In conclusion, both oral and subcutaneous D-gal showed significant beneficial effects on learning and memory, when its acute effects are taken into account. On chronic administration, beneficial effects were lost, and were comparable with the control or baseline values. On further chronic administration, there might have been complete worsening, as a tendency of decline was observed in the present study.

The effects of D-gal seem to vary with changes in the duration of therapy. Although it is routinely used to induce accelerated aging in rodents, there are studies that imply insufficient evidence for the same. Further studies may be required to ascertain the same, and also to confirm the beneficial effects produced with short-term use of the drug.

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