

Original Article

A simple and stable galactosemic cataract model for rats

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Abstract: Rat galactosemic cataract is commonly used in the investigation of sugar cataract. In current study, 21-day sprague-dawley (SD) rats were randomly divided into two groups (n=42), which were fed by normal water and galactose solution (12.5%-10%) for 18 days respectively. Every 3 days, lens opacity was observed by a slit lamp, and 6 rats of each group were executed for the analysis of aldose reductase (AR) activity, galactitol level and AR mRNA expression. Morphological results showed that small vacuoles initially appeared in the equatorial area before the 6th day, then subsequently extended to the whole anterior capsule, and eventually developed to mature cataract on the 18th day. AR of galactosemic lenses was significantly activated in the first stage and then slowly dropped to the end accompanied by the related changes of galactitol. AR mRNA expression also was upregulated and reached the peak at the 6th day. This study appears to confirm that galactosemic cataract can be induced for 21-day SD rats by only drinking 12.5% to 10% galactose solution, and this model is simple, economical and stable as to meet the research needs.

Keywords: Rat, galactose, cataract, model

Introduction

Diabetes patients are more easily to develop cataracts at a higher rate than normal persons. Its major characteristic is hyperglycemia. Three mechanisms are involved in sugar cataract formation: the polyol pathway, oxidation, and non-enzymatic glycation. The stimulation of polyol pathway is the important and relatively reversible pathologic process in the early stage, which has been extensively investigated.

AR, the key enzyme of polyol pathway, catalyzes the NADPH-dependent reduction of a wide variety of aldehydes and ketones, including glucose and galactose, to their corresponding alcohols [1, 2]. AR is only located in lens epithelial cells and differentiating epithelial cells with hypermetabolism in the equatorial area [1, 3]. Under the physiological condition, only a fewer proportion of glucose enters the polyol pathway. While, hyperglycemia gradually increase the glucose concentration in aqueous fluid, lens is dehydrated little by little due to the high osmotic pressure of glucose in aqueous fluid

[4, 5]. To diminish the further dehydration of lens, AR is heavily activated to produce an endogenous osmolyte sorbitol to increase lens osmolarity [6]. It's a part of the physiological osmoregulatory mechanism to cease or reserve the lens hydrated damage. Whereas sorbitol dehydrogenase activity is not simultaneously increased [7]. This shift on enzyme activity, combined with increased available lenticular glucose, favors sorbitol accumulation which badly affect osmotic imbalance with resultant influx of water. The hydration would be aggravated if blood glucose level drops suddenly, because it can magnify the osmotic gradient between lens cells and aqueous fluid [8]. The key role of AR has been demonstrated by the inhibition of AR inhibitors, which can effectively prevent sugar cataract formation in diabetic or galactosemic animal models [9-12].

There are differences between galactosemic and diabetic cataract. Galactose has higher affinity with AR than glucose; besides, galactitol is more difficult to be metabolized by sorbitol dehydrogenase than sorbitol. Hence, galactose-

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mia is more likely to produce severe sugar cataract in shorter time than hyperglycemia. As a result, galactosemic cataract has been commonly used as a model to investigate the mechanism or drugs on diabetic complications [11, 13-15].

In previous studies, rat galactosemic cataract was usually induced by 30% or 50% galactose diet [11, 14, 16-19]. This method is stable but costly. Besides, daily intraperitoneal injection of 30-50% galactose solution [20] or daily retrobulbarly injection of 20% galactose solution [21, 22] could also induce galactosemic cataract for rats. In both latter methods, the majority of rats had poor conditions and even died, and they were very unstable.

In this study, we want to introduce a stable and simple galactosemic cataract model just by feeding rats galactose solution.

Materials and methods

Animals

Male SD rats aged 20 days were purchased from the Institute of Laboratory Animal Science and housed two per cage with free access to standard chow. All the rats were treated according to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

Sugar cataract induced by gradient galactose solution

All the rats were randomized into two groups of 42, normal group had free access to normal water as control. The galactosemic group was given gradient galactose solution (Hanxi, China) as model, the galactose concentration was 12.5% for the first duration from day 1 to 7 and 10% for the second duration from day 8 to 18, and the galactose solution was changed and recorded daily. Eyes were examined by a portable hand-held slit lamp biomicroscope (Kowa, Japan) to detect the lens opacity every 3 days. Six rats of each group were randomly executed to dissect the intact lens immediately according to the posterior approach. Lenses were weighed and stored at -70°C for other analysis.

Ophthalmic examination of sugar cataract

Cataract severity was observed (without anesthesia) by a slit lamp proceeded by mydriasis

with topical administration of 0.1% tropicamide hydrochloride. Cataract was scored and recorded by an experienced observer who was blinded to identify of each lens in the time of assessing cataract severity. Initiation and development of lenticular opacity was graded into five categories as follows [12]:

Stage 0: clear lenses and no vacuoles present; Stage 1: vacuoles, located at the periphery of the lens, cover less than one third of the anterior of the anterior pole, forming a subcapsular cataract; Stage 2: vacuoles cover approximately two third of the lens anterior with/without the characteristic inverted-Y suture; Stage 3: some vacuoles have disappeared and the cortex exhibits a hazy opacity; Stage 4: a hazy cortex remained and dense nuclear opacity is present; Stage 5: a mature cataract is observed as a dense opacity in both cortex and nucleus.

AR activity and galactitol level in lenses [23-26]

Each lens was homogenized in 5 volumes of 50 mM ice-cold sodium phosphate buffer (pH7.2, with 5 mM 2-mercaptoethanol), then centrifuged at 15,000 g for 30 min at 4°C. The supernatant was collected to determine the lenticular AR activity, galactitol level and protein content.

AR activity was analyzed in 96 well plate based on the optical density value decrease of NADPH (Sigma, America) at 340 nm in 5 min at 25°C. The reaction mixture was in a total volume of 200 µl and consisted of 100 mM sodium phosphate buffer (pH 6.2), 0.4 M (NH₄)₂SO₄, 160 nM NADPH, 10 mM DL-glyceraldehyde (Sigma, America) as substrate and lens supernatant. The reaction was initiated by additional substrate at 25°C, lasted about 7 min and stopped at once by low temperature at -20°C. One unit of AR enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol NADPH per hour per 100 mg protein.

80 µL of lens homogenization was fully mixed with 20 µL of 20% TCA, centrifuged at 5,000 g for 10 minutes at 4°C, then collected the supernatant to determine the galactitol level. The reaction system consisted of the supernatant, 12.5 mM periodic acid, 12.5 mM sodium arsenite and 0.2% chromotropic acid. The absorption peak was recorded at 570 nm to calculate the level of galactitol.

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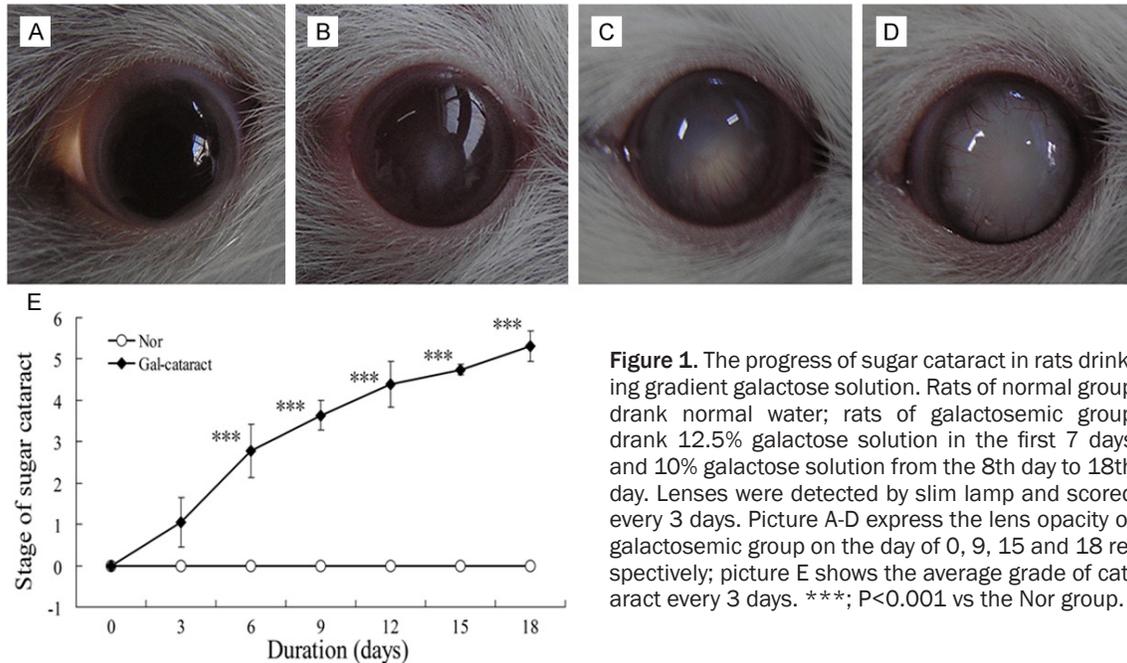


Figure 1. The progress of sugar cataract in rats drinking gradient galactose solution. Rats of normal group drank normal water; rats of galactosemic group drank 12.5% galactose solution in the first 7 days and 10% galactose solution from the 8th day to 18th day. Lenses were detected by slim lamp and scored every 3 days. Picture A-D express the lens opacity of galactosemic group on the day of 0, 9, 15 and 18 respectively; picture E shows the average grade of cataract every 3 days. ***; $P < 0.001$ vs the Nor group.

Protein concentration was determined according to the method of Bradford using bovine serum albumin as standard.

Real time RT-PCR analysis

The total RNA was isolated from the entire lens with Trizol reagent (Invitrogen, America). To avoid amplification of genomic DNA sequences, all RNA samples were treated with DNase I and diluted to 100 ng/ μ L. Then 2.5 μ g of total RNA of each sample was reverse-transcribed to the first strand cDNA with the iScript cDNA Synthesis Kit (Invitrogen, America). Quantification by real time RT-PCR was performed using SYBR Green PCR Kit (TaKaRa, China). All reactions were carried out in a single tube reaction setup on the ABI7000 real time PCR system (ABI, America). The following temperature profile was used: stage 1:10 s at 95°C for denaturation of cDNA/RNA hybrid, followed by stage 2: 40 cycles of 5 s at 95°C and 34 s at 61°C, and stage 3: 15 s at 95°C, 1 min at 60°C and 15 s at 95°C. β -actin, a housekeeping gene, was used as an internal control for PCR to normalize the expression of target genes. A control PCR reaction, involving taq polymerase and the primer combination, but without template, was used as a negative PCR control. Specific primers were designed based on published sequences (GenBank) and were as follows:

β -actin sense, 5'-ACT CTT CCA GCC TTC CTT C-3'; and anti-sense, 5'-ATC TCC TTC TGC ATC CTG TC-3'; AR sense, 5'-GTG ACC GAG GCT GTG AA-3'; and anti-sense, 5'-AGA GGG TTG AAG TTG GAG A-3'. The melting curve of each pair of primers was run to confirm that none of the primer pairs amplified additional bands or formed primer-dimers.

The cycle threshold (Ct) was used to calculate relative amounts of selected genes. Samples under the threshold line were considered to be below the background. The Cts of target genes were normalized to the level of β -actin as an endogenous control in each group. The average Ct for each gene was calculated by subtracting the Ct of the sample RNA from that of the control RNA for the same time measurement. This value was known as $\Delta\Delta$ Ct reflecting the relative expression of the treated sample compared to the normal control, and becomes the exponent in the calculation for amplification. Results were expressed as a fold change of target genes versus control gene as calculated by the $\Delta\Delta$ Ct method.

Statistical analysis

The cataract grade was analyzed by *Mann-Whitney test*, and other data were evaluated with one-way ANOVA (two-tailed test). The *P*

Galactosemic cataract model

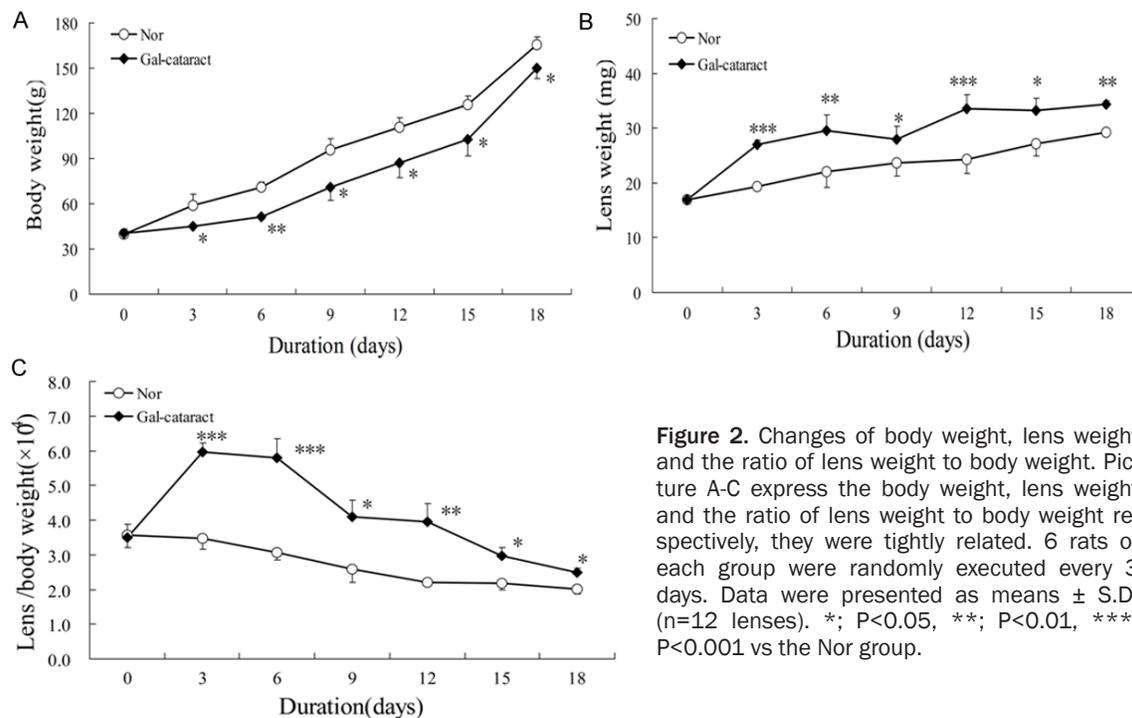


Figure 2. Changes of body weight, lens weight and the ratio of lens weight to body weight. Picture A-C express the body weight, lens weight and the ratio of lens weight to body weight respectively, they were tightly related. 6 rats of each group were randomly executed every 3 days. Data were presented as means \pm S.D. (n=12 lenses). *, P<0.05, **, P<0.01, ***, P<0.001 vs the Nor group.

value less than 0.05 was considered to be statistically significant.

Results

Onset and formation of rat galactosemic cataract

A nuclear cataract, developed in 21-day rats drank 12.5%-10% galactose solution, was easily visible to the naked eye as shown in **Figure 1**. **Figure 1A-D** vividly presented the lens opacity of galactosemic rats on the day of 0, 9, 15 and 18 respectively. The average grade of cataract was showed in **Figure 1E**. Lenses in normal group were all clear and at grade 0 during the entire period. On the contrary, the majority of lenses in model group developed cataract very quickly and the lens opacity gradually aggravated by one grade about every 3 days. In the early six days, small vacuoles appeared firstly at periphery, then subsequently extended to the whole anterior capsule, and sometimes formed the expanded inverted-Y suture in central area. On the 6th day, more than 85% of lenses were in stage 2, and only 5% still stayed in stage 1. From the 9th day, most lenses cortex showed hazy opacity and foggy nucleus. On the 15th day, lens nucleus had almost formed dense opacity, about 95% lenses were in stage

4, and only 3% were beyond stage 4. Eventually, over 95% lenses developed a full blown cataract on the 18th day (stage 5).

Effects of galactose solution on body weight, lens weight and the ratio of lens weight to body weight

Body weight of both groups increased gradually from the beginning to the end. Whereas, body weight of galactosemic group were always significant lower compared with normal group (P<0.05). Body weight of model group went up slowly in the first 6 days, and then experienced a marked increase in the latter. The gap between the two groups diminished at the end (**Figure 2A**).

At the same time, lens weight of two groups also increased consistently. At the beginning, the lens weight of the two groups is about 17 g. It is different that lens weight of galactosemic rat were always higher compared with normal level (P<0.05). The normal lens grew slowly up to 29.2 mg on the 18th day, but galactosemic lens grew up significantly in the early 3 days (27.07 mg), then continued to increase until the end (34.4 mg) though at a slow rate (**Figure 2B**).

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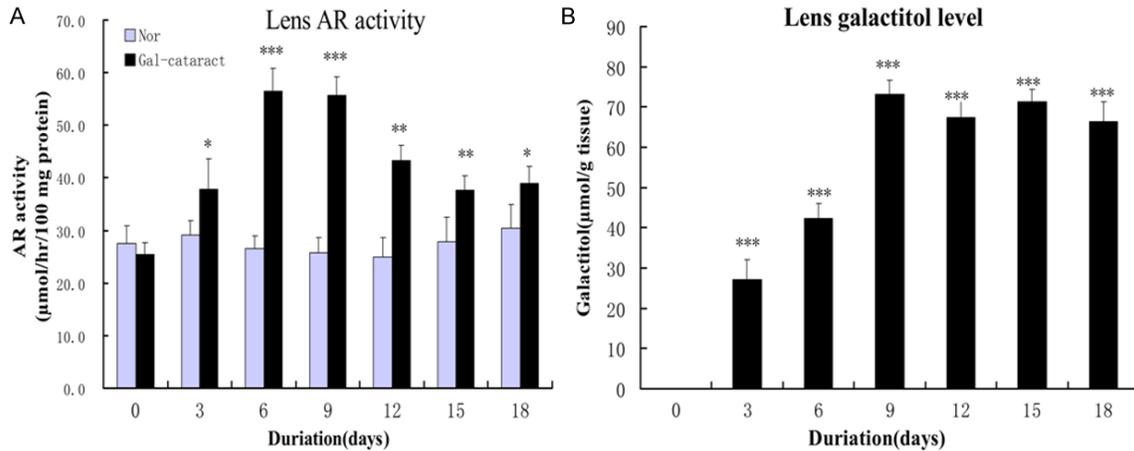


Figure 3. Effects of galactose solution on AR activity and galactitol level of lenses. A: AR activity. One unit is defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADPH per hour per 100 mg protein. Data were presented as means \pm S.D. (n=12 lenses). *, P<0.05, **, P<0.01, ***, P<0.001 vs the Nor group. B: galactitol level. Galactitol was almost undetected in normal lenses, Data were presented as means \pm S.D. (n=12 lenses). ***, P<0.001 vs that of galactose group on the day of 0.

As to the ratio of lens weight to body weight ($\times 10^{-4}$), the difference was greatest (**Figure 2C**). At the first glance, we could clearly see that the ratio of normal group decreased gradually between the day of 0 (3.58) to 18 (2.01), but the ratio of galactosemic rats sharply increased from the day of 0 (3.49) to 3 (5.97), leveled off until the day of 6 (5.81), then followed by a sudden decrease to the day of 9 (4.09), and continued to drop gradually to the end (2.49).

AR activity and galactitol level in lenses

As shown in **Figure 3A**, the specific activity of AR, a key enzyme of the polyol pathway, was always at the level of about 27.47 $\mu\text{mol/hr/100}$ mg protein in the whole process. But that of model group gradually ascended from the beginning (25.35 \pm 3.24) to the 3rd day (37.89 \pm 4.64), then remarkably reached the peak of 55.73 $\mu\text{mol/hr/100}$ mg protein on the 6th day, remained the level to the day of 9, and gradually decreased to 38.47 $\mu\text{mol/hr/100}$ mg protein at the end.

Additionally, galactitol level of lenses was also measured to identify the relationship of AR and polyol. The results showed that galactitol was almost undetected in normal lenses in the whole process, but it could reach 27.04 \pm 5.10 $\mu\text{mol/g}$ tissue in the galactosemic lenses on the 3rd day, sharply went up to 73.15 \pm 3.86 $\mu\text{mol/g}$ tissue on the day of 9, then slowly

dropped to the end (**Figure 3B**). Galactitol began to accumulate in the galactosemic lenses accompanied by the activated AR from the day of 6.

The expression of AR mRNA in the lenses of rat galactosemic cataract

To investigate the possible mechanisms of 12.5%-10% galactose solution induced cataract, we measured the AR mRNA expression related to the polyol pathway. Galactose drinking could evidently increase the expression of AR mRNA in the development of galactosemic cataract (**Figure 4**). AR mRNA expression was significantly upregulated and went up to the peak of 2.79 fold at the 6th day, then decreased slowly to 2.19-fold at the end.

Discussion

Cataract is one of the well-known secondary complications of diabetes and galactosemia. Both of them are together called "sugar cataracts". Numerous studies are ongoing by using various cataract models to investigate the cataractogenesis or crucial steps to halt this process. The galactosemic cataract model can rapidly mimic the pathological mechanisms of diabetic cataract [11, 13, 19].

For the first time, we found that only drinking 12.5%-10% galactose solution could signifi-

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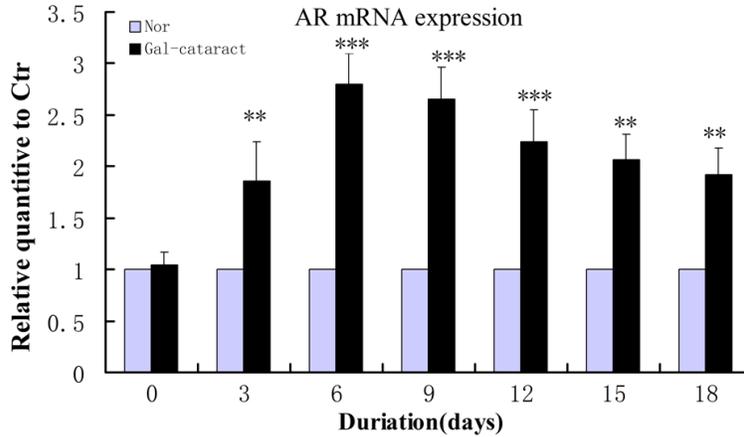


Figure 4. Real time RT-PCR analysis for the relative expression of AR mRNA in rat lenses. Data were presented as means \pm S.D. (n=12 lenses). **, P<0.01, ***, P<0.001 vs the Nor group.

cantly induce the stable sugar cataract for 21-day SD rats. The change of lens weight is exactly reverse with that of body weight. In the early period, body weight of galactosemic group increased very slowly, otherwise, their lenses weighed obviously heavier compared with normal level, so they together led to the higher ratio of lens weight/body weight. However, when the galactose solution was reduced to 10% from the 8th day, the body weight of galactosemic rats rose obviously, and lens weight increased slowly. In the present study, the changes of body weight and lens weight were tightly associated with the concentration of galactose solution. 12.5% galactose solution could greatly activate AR activity of lens, led to the accumulation of galactitol, and resulted in the hydration of lens. The slight increase of body weight was due to the hypergalactosemia of rats. Long-term drinking of galactose solution (12.5%-10%) produced a series of diabetic syndrome, plenty of water consumption, much urine and weight loss. Similar phenomenon was observed in streptozotocin induced diabetic cataract. No rats died during the entire experimental period of rat galactosemic cataract formation

From the results of slit lamp examination, we observed that a large number of dense clusters of vacuoles located in the equatorial area before the 6th day, and then subsequently expanded to the anterior capsule, obviously flocculent turbidity appeared in the lens cortex by the day of 12. In general, it took about 18

days to form the mature sugar cataract, which was so rapid and economical to fulfill the need of research. Findings of 5 times experiments showed the successful rate of rat galactosemic cataract was over 95%. Besides, biochemical results suggested that high level galactose could activate the existing AR to produce plenty of galactitol. The accumulation of galactitol was accompanied by the gradual increase of AR activity and lens hydration.

The hydration of galactitol in lens formed small vacuoles, which triggered the osmotic expansion of the lens. All these changes were caused by 12.5% galactose solution in the early stage. After the 6 days, we found that the rats showed polydipsia and polyuria. To relieve the current condition, we decreased the galactose concentration from 12.5% to 10% from the 8th day, and noticed that the status of rats was obviously improved, body weight gradually increased and the lens opacity were getting worse and worse.

Galactose concentration is critical in the development of rat galactosemic cataract. We found that if rats drank 10% galactose solution from the beginning, the successful rate would greatly reduced. And if we raised the galactose concentration to 15% from the beginning, the lens osmotic pressure was too high to maintain good status of rats, and even caused the death. The galactose concentration here used had already been confirmed through many times of strict experiments. Rats' status would be good if the galactose concentration was timely adjusted.

Rat age was same important as galactose concentration in the successful establishment of rat galactosemic cataract model. Younger animals were more sensitive to galactose than elder, so we chose the 21-day weanling rats. If rats used were over 30 days, and still drank the 12.5%-10% galactose solution as before, the sugar cataract model was almost unable to be successfully formed. In this study, we should pay more attention to the two key aspects, one

is rat age and another is galactose concentration, both of them must be tightly combined together.

Conclusion

In summary, galactosemic cataract can be successfully developed in 21-day SD weanling rats by only drinking gradient galactose solution (12.5%-10%). This method is so rapid, stable and convenient that it would be widely used by researchers to investigate the sugar cataract mechanism or screen a variety of candidates with anticataract potential.

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Disclosure of conflict of interest

None.

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