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**ORIGINAL ARTICLE****Randomized Controlled Trial**

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Randomized Controlled Trial

Anticataractogenic effect of hesperidin in galactose-induced cataractogenesis in Wistar rats

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Abstract

AIM: To explore the anticataractogenic potential of hesperidin, a flavanone, in galactose-induced cataractogenesis.

METHODS: In this study, cataract was induced by administering galactose enriched food in a set of rats. Effect of different dosages of hesperidin (25, 50 and 75 mg/kg body weight) were administered simultaneously with galactose in prevention of cataract was determined in another set. In both sets of animals, the levels of peroxidation, oxidants (NO and OH), antioxidants (enzymatic: Superoxide dismutase, catalase, glutathione S-transferase, GPx and non-enzymatic: Reduced glutathione, vitamin E), aldose reductase and sorbitol were determined in the eye lens. In addition, glucose and lipid peroxidation levels were also tested in serum. The quantitative changes in lens inducible nitric oxide synthase (iNOS) and its expression were also determined using Western blot and real-time polymerase chain reaction analyses.

RESULTS: Galactose enriched food produced cataract in both the eye lens as a sequel to elevated serum glucose. Simultaneous administration of hesperidin not only reduced serum glucose but also prevented cataract development, through reduced levels of reactive oxygen species (NO and OH) and iNOS expression as well as elevated enzymic and non-enzymic antioxidants were observed in the eye lens.

CONCLUSION: These results indicate the preventive

effect of hesperidin against cataract in hyperglycemic rats.

Key words: Antioxidants; Oxidative stress; Galactose-induced cataract; Free radicals; Hesperidin; Eye lens

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Core tip: Hesperidin acts as an anticataractogenic agent in preventing development of cataract upon galactose induction in rats. At all the doses tested, hesperidin was able to prevent deleterious changes caused by galactose in eye lens.

Manikandan R, Arumugam M. Anticataractogenic effect of hesperidin in galactose-induced cataractogenesis in Wistar rats. *World J Ophthalmol* 2016; 6(1): 1-9 Available from: URL: <http://www.wjgnet.com/2218-6239/full/v6/i1/1.htm> DOI: <http://dx.doi.org/10.5318/wjo.v6.i1.1>

INTRODUCTION

Diabetes, a multifactorial metabolic dysregulation, is fast becoming one of the dreaded diseases. Secondary complications that arise due to diabetes further adds to the disease burden and severity^[1,2]. Among the secondary complications, diabetic cataract is one of the major concerns that if left undetected can cause profound blindness^[1]. In a survey conducted during December 2010 in India, it was found that 77.5% of all avoidable blindness was contributed by cataract. The incidence was found to be higher (66%) in diabetic patients^[3]. Pathogenesis of diabetic cataract is well known to involve damage to lens constituents, increased accumulation of polyols like (galactitol, sorbitol) and osmotic stress. These lead to swelling of eye lens and disruption in the native state of proteins^[4-6]. In lens, hyperglycemia due to diabetes causes the activation of polyol pathway increasing incidence of cataract^[6-8]. Sorbitol, forms the first step in polyol pathway and is a major contributor of osmotic stress. In addition, fructose, a breakdown product acts as a potent radical generator^[9]. Together, these changes can contribute to diabetic-cataract. Other studies on galactose-induced cataract has shown decrease in lens glutathione^[10], lens epithelial cell apoptosis^[11] and even inhibition of cell cycle progression^[12].

Apart from surgical removal of cataractous lens, there are no therapeutic strategies available in the case of diabetic-cataract. However, cataract surgery, though common and relatively safe, is not completely risk free^[13]. Irreversible blindness is one major concern and moreover, any surgery especially for patients with diabetes is complicated. Thus natural or plant-based interventional strategies are emphasised for cataract

prevention as they do not have adverse side effects and are easily accessible. Various studies including some of ours^[14-17] have indicated the potential of plant based compounds as effective anticataract agents^[17].

Hesperidin, a flavanone glycoside and an antioxidant, is found abundantly in citrus fruits^[18]. Studies in humans and animals have shown the importance of hesperidin in cholesterol reduction^[19], blood pressure^[20], prevention of loss in bone density^[21], inhibition of cell cycle progression^[22], antiviral^[23] and radical scavenging activities^[24]. Another flavanoid, resveratrol was previously shown to possess anticataract activity against selenite-cataract^[25], however, the protective mechanism of such flavanone, against cataract is still unclear. Thus, to our knowledge this study depicts the potential and efficiency of hesperidin against galactose-induced oxidative stress leading to cataract in rats.

Our primary investigation was to determine whether hesperidin administration could prevent development of galactose-induced cataract formation by monitoring various oxidative stress parameters and lens antioxidants.

MATERIALS AND METHODS

Experimental conditions

Male Wistar albino rats weighing about 100-150 g of body weight were randomly allocated into five groups with six animals each. They were housed in separate cages under standard temperature ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and 12-h light/dark photoperiod. They were acclimatized for a week before initiation of the experiment, provided food and water *ad libitum*. All experiments were designed and conducted according to the guidelines of committee for the purpose of control and supervision on experiments on animals.

Galactose administration

Thirty percent of galactose was given to rats in their daily food for one month^[26].

Treatment with hesperidin

Hesperidin prepared in gum acacia (1%) was administered daily by intragastric intubation for a month. Three concentrations of hesperidin (25, 50 and 75 mg/kg body weight) were tested in this study.

Animals were grouped in to 5 with 6 in each group:

The effective dose of hesperidin was determined by performing dose-response experiments. Group I : Control, Groups II to V different concentrations of hesperidin 25, 50, 75 mg/kg body weight simultaneously were administered to the rats instilled with galactose (30%).

Cataract detection

Animals from each group were monitored for cataract formation periodically using slit lamp technique as described by^[27].

Tissue preparation

At the end of this experiment rats were given pentobarbital (50 mg/kg). Eye lens were removed, rinsed using ice-cold saline and stored at -70°C till further analyses. In the case of hydroxyl radical estimation after washing lens were used immediately. Before analyses, the lens were homogenized in ice-cold 10% Tris-buffer (0.1 mol/L, pH 7.2), centrifuged (12000 rpm, 30 min, 4°C) and supernatant used. Blood was also collected from animals before sacrifice from tail vein and harvested serum. Animals were weighed once a week. However, body weights of rats did not show any significant difference between groups. They did not show any weight gain or loss from the beginning of experiment till the end (data not shown).

Biochemical estimation

Glucose and lipid peroxidation were estimated as described by^[28] and^[29], respectively.

Antioxidant

Quantitative analyses of eye lens antioxidants:

Tissues were prepared as previously described and protein level was quantified as in^[30]. Antioxidant enzymes such as catalase (CAT)^[31], superoxide dismutase (SOD)^[32], GPx^[33], glutathione (GSH)^[34], glutathione S-transferase (GST)^[35] and vitamin E^[36] were estimated spectrophotometrically using eye lens homogenates.

Quantitative analysis of free radicals in eye lens

Nitric oxide^[37] and hydroxyl radical^[38] generations were estimated in the eye lens.

Western blot analysis and real-time polymerase chain reaction

Separation of eye lens homogenate was executed electrophoretically by the method elaborated in^[39]. The Western blot analysis was systematically performed as described in while real-time polymerase chain reaction (RT-PCR) was done as explained in^[16,40].

Lens aldose reductase and sorbitol measurements

Aldose reductase and sorbitol levels were estimated as described by^[41] and^[42], respectively.

Statistical analysis

Student's *t*-test was done between data of different groups expressed as mean \pm SD. The statistical treatments were affirmed by a biomedical statistician.

RESULTS

Cataract formation

Thirty percent of galactose administration to rats for 30 d resulted in cataract formation in Group II animals. Out of six animals four showed cataract formation while in two there were no signs of cataract. In the case of hesperidin treatment we did not observe cataract in any of the

animals belonging to Group IV (50 mg/kg) and V (75 mg/kg). Surprisingly, in Group III (25 mg/kg) only one animal out of six developed cataract.

Note: Comparison of all experimental data was done between various groups under two main categories: Groups II and I (Statistical significance $P < 0.05$); Groups III, IV and V with II (Statistical significance $P < 0.001$ or 0.05).

Serum glucose (Table 1) lipid peroxidation (Figure 1), nitric oxide and hydroxyl radical generation (Table 2) increased in Group II, while it decreased in III, IV and V.

Antioxidant activity of eye lens

Enzymic (SOD, CAT, GPx, GST), and non-enzymic antioxidants (GSH and vitamin E) levels were found to be decreased in Group II while increased in III, IV and V animals (Tables 3 and 4).

Aldose reductase and sorbitol were found to be increased in II animals but decreased in III, IV and V animals (Table 5).

Western blot analysis

Western blot data depicted a fraction of approximately 130 kDa and inducible nitric oxide synthase (iNOS) expression was higher in Group II (lane II), whereas in Groups III, IV and V it was noted to decrease significantly (Figure 2A). RT-PCR result too was recorded similar to blot data (Figure 2B).

DISCUSSION

Diabetes is a multifactorial metabolic disorder that apart from causing hyperglycemia, more often leads to widespread changes involving multiple organ dysfunction and failure. It is thus ironical that diabetes can be only managed under current clinical settings^[43]. Nevertheless, it is the secondary complication associated with diabetes that are highly debilitating and cataract is one among them^[1]. Pathogenesis of diabetic cataract is well known to involve increased accumulation of polyols like galactitol and sorbitol, causing osmotic stress. These leads to swelling of eye lens, disruption in the native state of proteins and hence causes opacity^[6]. Hyperglycemia specifically triggers the polyol pathway consisting of enzyme aldose reductase that breaks down excess glucose^[6]. Fructose, a breakdown product, is another culprit^[9]. Taken together, sorbitol-induced osmotic stress and fructose-generated free radicals alter normal lens physiology leading to cataract formation. In addition, there is the pathology associated with advanced glycation end products^[44,45] due to hyperglycemia that can potentially contribute to disruptions in cellular signalling. Thus it is obvious that aldose reductase inhibitors can prove beneficial; however, there are reports of deleterious side effects and lack of appropriate action with these inhibitors^[46,47]. In line with this, flavanoids such as hesperidin too have been shown to inhibit aldose reductase^[48], but its efficacy against galactose-induced

Table 1 Effect of hesperidin on glucose profile in the serum of normal and treated rats

Enzymes analyzed (unit of activity)	Group I	Group II	Group III	Group IV	Group V
Glucose (mg/dL)	122.36 ± 2.55	191.53 ± 4.69 ^a	166.52 ± 5.47 ^b	134.73 ± 4.89 ^b	122.86 ± 4.52 ^a

Each value represents the mean ± SD of observation made on samples from six animals from the same group. Statistical analysis was performed by the student *t*-test. Letter “a” and “b” indicate that the difference observed between the Groups I and II or between III, IV, V or are statistically significant at ^b*P* < 0.001 and ^a*P* < 0.05.

Table 2 Effect of hesperidin on free radical generation in the eye lens of albino rats exposed to galactose

Free radical analyzed (unit of activity)	Group I	Group II	Group III	Group IV	Group V
Nitrite (nmol/g wet weight)	13.19 ± 1.69	36.00 ± 5.49 ^a	29.58 ± 3.13 ^b	22.11 ± 3.84 ^b	14.62 ± 3.29 ^a
Hydroxyl radical (OD 510 nm/30 min)	1.84 ± 0.46	4.78 ± 1.58 ^a	3.41 ± 0.59 ^b	3.08 ± 0.80 ^b	2.24 ± 0.39 ^a

Each value represents the mean ± SD of observation made on samples from six animals from the same group. Statistical analysis was performed by the student *t*-test. Letter “a” and “b” indicate that the difference observed between the Groups I and II or between III, IV, V or are statistically significant at ^b*P* < 0.001 and ^a*P* < 0.05.

Table 3 Quantitative analysis of enzymatic antioxidants in eye lens of albino rats

Enzymes analyzed (unit of activity)	Group I	Group II	Group III	Group IV	Group V
Superoxide dismutase (units/mg protein)	8.59 ± 1.02	3.29 ± 0.67 ^a	3.95 ± 0.68 ^b	5.51 ± 0.95 ^b	7.90 ± 0.62 ^a
Catalase (μmol H ₂ O ₂ consumed/mg protein/min)	21.16 ± 3.02	10.59 ± 2.12 ^a	12.75 ± 3.42 ^b	14.4 ± 3.45 ^b	18.93 ± 3.76 ^a
Glutathione peroxidase (μmol glutathione oxidized/mg protein/min)	76.80 ± 5.44	38.81 ± 6.61 ^a	50.05 ± 7.90 ^b	61.50 ± 3.43 ^b	69.82 ± 4.19 ^a
Glutathione-S-transferase (μmol H ₂ O ₂ consumed/protein/min)	13.20 ± 2.44	6.03 ± 1.00 ^a	7.41 ± 1.01 ^b	8.96 ± 1.60 ^b	11.44 ± 1.12 ^a

Each value represents the mean ± SD of observation made on samples from six animals from the same group. Statistical analysis was performed by the student *t*-test. Letter “a” and “b” indicate that the difference observed between the Groups I and II or between III, IV, V or are statistically significant at ^b*P* < 0.001 and ^a*P* < 0.05.

Table 4 Quantitative analysis of non-enzymatic antioxidants in eye lens of albino rats

Enzymes analyzed (unit of activity)	Group I	Group II	Group III	Group IV	Group V
Reduced glutathione (μmol/g wet)	8.37 ± 1.78	3.77 ± 1.04 ^a	4.06 ± 1.48 ^b	4.93 ± 0.76 ^b	7.24 ± 0.91 ^a
Vitamin E (μg/mg protein)	3.79 ± 0.84	1.34 ± 0.41 ^a	1.68 ± 0.27 ^b	2.03 ± 0.13 ^b	2.79 ± 0.50 ^a

Each value represents the mean ± SD of observation made on samples from six animals from the same group. Statistical analysis was performed by the Student *t*-test. Asterisks indicate that the difference observed between the Groups I and II or between III, IV, V or are statistically significant at ^b*P* < 0.001 and ^a*P* < 0.05.

Table 5 Aldose reductase activity and sorbitol levels in the eye lens of albino rats

Groups	Lens aldose reductase activity (nmol NADPH/min per milligram protein)	Lens sorbitol (μmol/L per gram)
I	19.67 ± 2.05	0.18 ± 0.001
II	28.34 ± 1.48 ^a	2.0097 ± 0.008 ^a
III	26.62 ± 2.22 ^b	1.77 ± 0.005 ^b
IV	24.18 ± 1.34 ^b	1.43 ± 0.003 ^b
V	20.25 ± 1.69 ^a	0.938 ± 0.007 ^a

Each value represents the mean ± SD of observation made on samples from six animals from the same group. Statistical analysis was performed by the Student *t*-test. Asterisks indicate that the difference observed between the Groups I and II or between III, IV, V or are statistically significant at ^a*P* < 0.001 and ^b*P* < 0.05.

cataract is unknown.

Due to the lack of therapeutic drugs against cataract,

emphasis is being laid on cytoprotective strategies and in recent years traditional medicines, primarily derived from plant sources is gaining prominence. With regard to cataract, studies including ours, have demonstrated the potential of plant-based compounds that particularly possess antioxidant and anti-inflammatory activities as effective anticataract agents^[14-17]. Hesperidin is one such promising plant-based compound^[19-24], however to our knowledge it has never been tested for its activity against cataract. Galactose-induced cataract in animals is well established and the use of 30% dietary glucose for inducing cataract is accepted, for several investigations into hyperglycemia related cataract in humans^[26].

Consistent with these earlier studies, our results showed that administration of 30% galactose leads to cataract formation in four out of six animals (Group II). Two of the animals failed to show cataract formation and we assume a robust physiological adaptation by these two

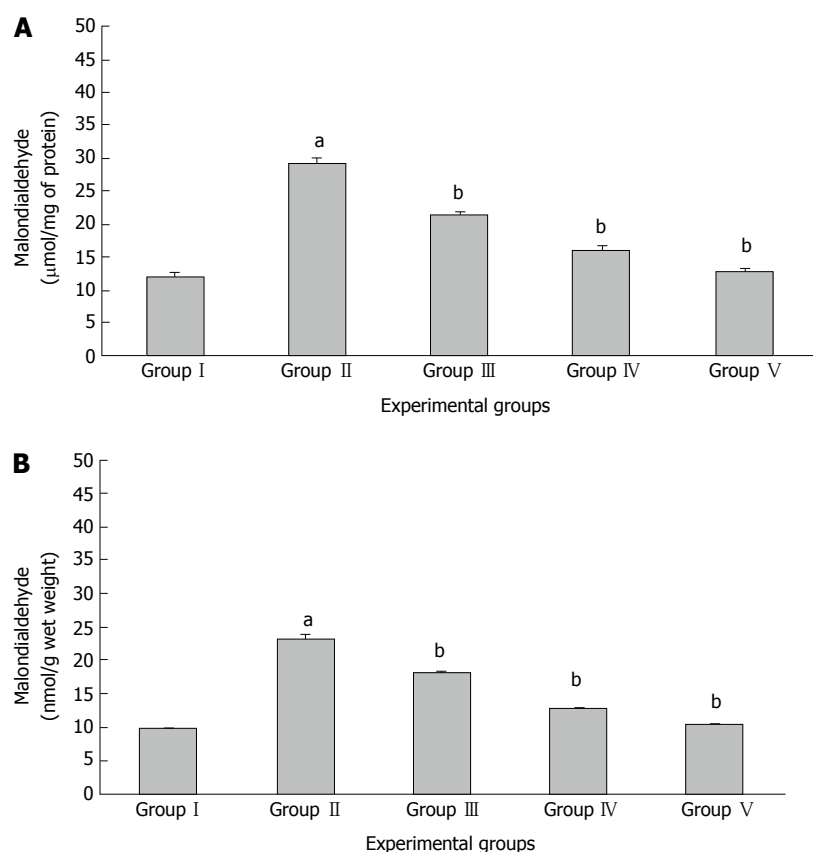


Figure 1 Quantitative analysis of malondialdehyde in the serum of Wistar albino rats (A) and in the eye lens of Wistar albino rats (B). Each value represents the mean \pm SD of observations made on samples from four determinations from the same group. Statistical analysis was performed by the student's *t*-test. Letter "a" and "b" indicate that the difference observed between Group I and II and Group II and III-V animals were statistically significant at $^bP < 0.001$ and $^aP < 0.05$.

animals in response to increased galactose administration. On comparison with Group I, supportingly, the serum glucose of Group II animals was also found to be significantly elevated. However, upon hesperidin treatment (25, 50 or 75 mg/kg) simultaneously with 30% galactose for one month, it lead to a complete absence of cataract in animals belonging to Group IV and V. Surprisingly, one animal out of six in Group III (25 mg/kg hesperidin) developed cataract and at this juncture we do not have any explanation for this. In line with these results, hesperidin had a significant hypoglycaemic effect^[49] in animals of Groups II, IV and V than II.

Lipid peroxidation is a direct indicator of free radical induced membrane damage and this can have drastic consequences on lens membrane proteins. Free radical induced lipid peroxidation was found to be elevated in serum and eye lens from Group II animals indicating sugar induced osmotic stress leading to free radical generation^[25,50]. In the presence of hesperidin (Group III, IV and V), levels of lipid peroxidation (LPO) in both serum and eye lens were reduced implicating the antioxidant nature of hesperidin. With regard to lens lipid peroxidation, our earlier study with curcumin^[16] has shown that lens LPO can cause changes in Ca^{2+} ATPases, leading to disruption in lens Ca^{2+} homeostasis. Under these conditions, curcumin was able to rescue lens Ca^{2+} ATPases from free radical induced damage and these

animals showed complete absence of cataract^[16].

Both nitric oxide and hydroxyl radicals were found to be highly elevated in eye lens of rats administered with galactose alone, suggesting enhanced oxidative stress mediated by these two radicals. Their generation could primarily contribute to oxidative stress, increased LPO and leads to cataract development in animals exposed to galactose alone. Indeed, hydroxyl radical has been shown to trigger LPO and oxidation of non-SH groups in lens^[51]. Similarly, increased nitric oxide in the eye lens affects free protein thiols and total glutathione levels^[52] leading to lens opacification^[16]. Use of hesperidin lead to a significant decrease in lens nitric oxide and hydroxyl radical generation suggesting the efficacy of hesperidin to neutralize these free radicals^[24,25] in eye lens and prevent cataract. Supportingly, lens iNOS expression was found to be elevated in galactose alone administered animals (Group II), while the expression was found to be reduced or almost comparable to that of control (Group I) in case of hesperidin treated animals (Group III, IV and V). This clearly shows the ability of hesperidin to modulate iNOS expression in eye lens.

Antioxidant defences comprise agents that catalytically remove free radicals^[53] and preserve the oxidative status of a cell. In this study, a series of enzymic and non-enzymic antioxidants were analysed in eye lens to understand the effect of hesperidin on their activities.

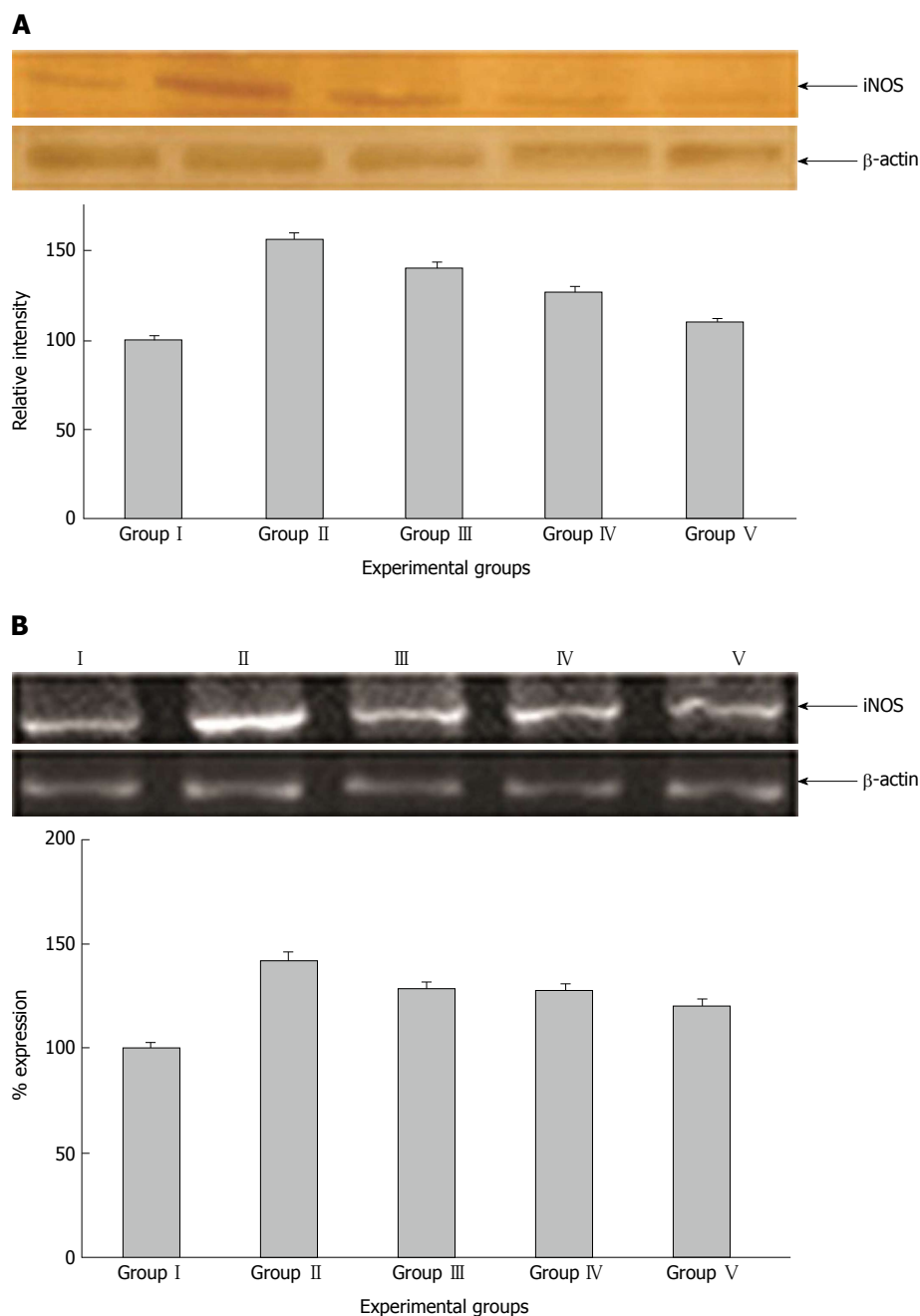


Figure 2 Immunoblot expression of inducible nitric oxide synthase in control and experimental group of animals (A) and effect of hesperidin on inducible nitric oxide synthase gene expression in the eye lens of Wistar rats exposed to galactose (B). A: Lane I, eye lens protein from control (physiologic saline) rats (Group I); lane II, eye lens protein from galactose administrated rats (Group II); lane III, eye lens protein from animals administered with galactose and 25 mg/kg body weight hesperidin simultaneously (Group III); lane IV, eye lens protein from animals administered with galactose and 50 mg/kg body weight hesperidin simultaneously (Group IV); and lane V, eye lens protein from animals administered with galactose and 75 mg/kg body weight hesperidin simultaneously (Group V). The separated lens protein was preincubated with anti-iNOS polyclonal IgG antibody (1:500 dilution) and subsequently with goat antirabbit IgG-HRP (1:3000 dilution). The immunoreactivity was developed with 0.01% DAB and H₂O₂. β-actin refers to housekeeping protein expression and its levels are constant across all treatment groups indicating the normal behaviour of lenses under various treatment. The figure clearly shows increased iNOS protein expression under galactose mediated oxidative stress. This increased iNOS protein expression was prevented by hesperidin in a dose dependent manner; B: Lane I, mRNA expression in lens from rats treated with saline alone (Group I); lane II, mRNA expression in lens from rats administered with galactose alone (Group II); lane III, mRNA expression in lens from rats administered with galactose and hesperidin 25 mg/kg body weight simultaneously (Group III); lane IV, mRNA expression in lens from rats administered with galactose and hesperidin 50 mg/kg body weight simultaneously (Group IV); lane V, mRNA expression in lens from rats administered with galactose and hesperidin 75 mg/kg body weight simultaneously (Group V). Galactose-mediated oxidative stress causes an increase in the expression of iNOS gene, which probably underlies the pathogenesis of cataract induced by galactose. All these changes were prevented by hesperidin simultaneously, indicating its protective effect. iNOS: Inducible nitric oxide synthase.

SOD dismutates superoxide anion and prevents initiation of free radical chain reaction. Previously our studies too have implicated the importance of SOD in lens^[15,16].

Catalase, a haem containing redox enzyme found in eye lens catalyzes the conversion of H₂O₂ to water and molecular oxygen. GPx reduces lipid hydroperoxides

and free H₂O₂ to their corresponding alcohols as well as water. GST are proteins with multiple functions, which can detoxify electrophilic compounds and protects from ill-effects of peroxidase^[54]. It is quite obvious that under high oxidative stress these antioxidants are overwhelmed leading to cellular damage. Significant decrease in all the enzymic antioxidants was observed in eye lens from Group II animals than I. This clearly suggests oxidative stress triggered by hyperglycemia in eye lens, could have caused cataract formation. However, administration of hesperidin protected these antioxidants from galactose induced oxidative stress and thus prevented cataract formation.

Reduced glutathione is found at high levels in eye lens, which maintains reduced state of protein thiol groups and prevents cross linking of soluble crystallins of lens^[55]. GSH scavenges free radicals, serves as co-substrate for GPx activity, as a cofactor for many enzymes and forms conjugates in endo as well as xenobiotic reactions^[56]. Galactose administration has been shown to reduce GSH level in lens due to increased lipid peroxidation^[57], which supports the results in our present study. As observed with enzymic antioxidants, presence of hesperidin rescued lens GSH that could have potentially contributed to its anticataract action. Vitamin E is an antioxidant that can inhibit lipid peroxidation, help preserve membrane integrity and vital membrane functions^[58]. Studies have also shown the ability of vitamin E in preventing cataract, including galactose-induced cataractogenesis^[59,60]. In this report, vitamin E was significantly decreased in Group II animals, whereas, administration of hesperidin along with galactose completely brought the levels of lens vitamin E back to normal. In addition, hesperidin treatment was shown to inhibit lens aldose reductase activity as well as lens sorbitol accumulation. Sorbitol accumulation can lead to osmotic stress that can in turn cause oxidative stress. By inhibiting aldose reductase activity hesperidin reduced sorbitol accumulation in lens and thus protects lens from cataract formation.

To conclude our report demonstrate that hesperidin can function as an anti-cataract agent against galactose-induced cataract in rats. Interestingly, all the doses of hesperidin tested prevented galactose-induced deleterious changes in eye lens and thus prevented cataract formation. This study gives proof to yet another important function of hesperidin and more work is needed to understand the exact anticataract action of hesperidin for possible future applications.

COMMENTS

Background

Hesperidin, a plant derived flavanone, is being used to prevent/control or regulate various oxidant mediated diseases including cancer, nephrotoxicity and hyperglycemia in adult rats. Similarly, a variety of phenolic or flavanones such as curcumin, ferulic acid or resveratrol are known to prevent selenium-induced (oxidant) cataract development in rat pups.

Research frontiers

In this study, the authors attempted to assess the potentials of hesperidin in

prevention or regulation of hyperglycemic mediated cataract development in adult rats.

Innovations and breakthroughs

The results of this study clearly indicate that hesperidin not only regulates hyperglycemia but also prevents development of cataract in adult rats.

Applications

These results opens up newer researches in developing plant derived non-toxic compound with multiple functions against complex diseases such as hyperglycemic mediated oxidative injuries to the susceptible tissues/organs.

Peer-review

This is an interesting paper investigating the anticataract properties of hesperidin against galactose-induced cataract in rats. The author is focused on the ability of hesperidin to attenuate the galactose-induced oxidative stress which is a major pathogenetic mechanism of galactose cataract. Therefore, the author determined a wide panel of oxidative stress biochemical markers and tested their modulation by hesperidin treatment.

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