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Research Article

PANCREATOPROTECTIVE EFFECT OF SILYMARIN ON OXIDATIVE STRESS IN ALLOXAN-INDUCED HYPERGLYCEMIA IN MALE WISTAR RATS

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ABSTRACT

Objective: The objective of the study was to study the silymarin's pancreatoprotective effect in alloxan-induced Type I diabetes mellitus. Numerous studies have evidence to prove the fact that antioxidant defense mechanism of flavonoids has overcome the progression of chronic diabetic complications.

Methods: A total of 24 male Wistar rats were divided into four groups (n=6): Group I normal control, Group II, Group III, and Group IV were induced diabetes with alloxan. Group I and Group II diabetic rats received the vehicle (PO). Group III was treated with silymarin 400 mg/kg (PO). Group IV was treated with glibenclamide 0.5 mg/kg, per orally for 21 days. Fasting blood samples were collected from all four groups of animals at the end of 21 days to evaluate serum glucose and glycosylated hemoglobin (HbA1c). Pancreatic tissue extraction, to perform lipid peroxidation and histopathological study confirms the level of oxidative damage to tissues and recovery after treatment.

Results: The serum glucose and HbA1c levels significantly increased in untreated diabetic rats, also a significant rise in lipid peroxidation and necrosis of beta cells in the pancreatic tissue. The rise in serum glucose levels was ameliorated in rats treated with silymarin, pancreatic tissue showed increased antioxidant levels, decreased lipid peroxides, and minimal changes and signs of regeneration of beta cells.

Conclusion: This study adds experimental evidence to the fact that silymarin is an effective nutritional supplement to treasure pancreatic beta-cell reserve and to delay diabetic complications.

Keywords: Diabetes, Oxidative stress, Pancreas, Silymarin.

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INTRODUCTION

Diabetes mellitus (DM) is one of the common metabolic disorders characterized by increased blood glucose levels (hyperglycemia) which is associated with carbohydrate, fat, and protein metabolism abnormalities and leads to chronic micro- and macro-vascular complications [1]. According to American Diabetes Association [2], Type II DM is associated with relative insulin deficiency and insulin resistance, beta-cell destruction with absolute insulin deficiency leads to Type I DM [3].

According to IDF Atlas 2015 [4], the estimated number population with diabetes worldwide in 2040 will be 642 million. A constant high glucose level in people with diabetes leads to the risk of developing countless disabling and life-threatening health problems. In India, 926 million in 2015 are diagnosed with DM and estimated number in 2040 will be 1.31 billion [5].

Hyperglycemia leads to increased production of reactive oxygen species (ROS) in mitochondria. Various animal studies [6,7] have proved that the oxidative stress due to hyperglycemia causes a reduction in beta-cell mass and impairment in its function. Kaneto *et al*. [8] reported that in addition to reduction in antioxidant enzymes, beta cells exposed to high glucose concentration also showed advanced glycosylated end products.

Various pre-clinical trials and animal experimental models proved that dietary antioxidants supplementation [9] has reduced abrupt incidence disorders due to tissue oxidative damages. Flavonoids can exert [10] antioxidant mechanisms by various mechanisms, for example, by scavenging free radicals, or by inhibiting the enzymatic systems responsible for free radical generation.

Silybum marianum L. (milk thistle) is a medicinal plant from the ancient period, which has been used for more than 2000 years for the treatment of liver and gall bladder disorders [11-13]. Silymarin an active component of this herb has gained attention due to its other beneficial activities [14] such as anticancer, and cytoprotection. These include [15-17], stimulation of ribosomal RNA polymerase in tissues, enhanced stimulation of hepatocyte regeneration, with pro-apoptotic, and antiangiogenic effect.

Hence, in this present study, the cytoprotective effect of silymarin which had been proved consecutively in various tissues (liver, kidney, etc.,) in numerous studies, is now proved to be an effective barrier against the destruction of pancreatic islet cells and also the regeneration of the pancreatic islet cells were depicted with suitable examples. Studies on pancreatic tissues are less with silymarin utilization as a cytoprotective agent. This study was done to explore the pancreatoprotective effect of silymarin against hyperglycemia-induced oxidative stress in male Wistar diabetic rats.

METHODS

This animal experiment was undertaken in the central animal house of Rajah Muthiah Medical College and Hospital (RMMCH), Annamalai University, Chidambaram, in accordance with National Institute of Health (1985) "guide for the care and use of laboratory animals." The animal experiment was approved by the Institutional Animal Ethics Committee of Annamalai University (No.160/1999/CPCSEA), Tamil Nadu, India. The proposal No.1017 was approved on May 2, 2013).

Duration of the study
This duration of the study was 6 weeks.

Experimental animals
Healthy male Wistar rats weighing 230–250 g were included in the study. A total of 24 male Wistar rats from the central animal house, RMMCH, Annamalai University in Tamil Nadu were utilized in the study. The male Wistar rats were housed in polypropylene cages (28 cm×22 cm×14 cm) bedded with husk, six per cage, under controlled environmental conditions (temp-23±2°C, humidity 65–70%, and 12 h light/dark cycles) at the central animal house, housed for at least 7 days in central animal house and then 2 h in laboratory before the commencement of experiment. All experiments were performed during the light phase of cycle. Animals were fed with standard pellet diet (VRK Nutritional Solutions, Baramati Agro Limited, Sangli Maharashtra, India) and water *ad libitum*.

Chemicals and reagents used
- Silymarin (milk thistle) – from nature's bounty Inc., Bohemia. NY 11716 U.S.A
- Alloxan monohydrate (2,4,5,6-tetraoxypyrimidine-2,4,5,6-pyrimidinetetrone) – from MP Biomedical India Private Limited, Mumbai, Maharashtra
- Biomedical and enzymatic kits were obtained from Sigma-Aldrich Chemicals and Mouli Enterprises
- glycosylated hemoglobin (HbA1c) was determined using HbA1c kit, purchased from Mouli Enterprises, Pondicherry
- Blood glucose was analyzed using glucometer, obtained from Srinivasan Enterprises, Madurai.

Preparations and dosage calculations
- Silymarin solution
  Silymarin powder+distilled=50 mg/ml silymarin solution.
- Glibenclamide solution
  Glibenclamide tablets+distilled water=0.5 mg/kg glibenclamide solution [18,19] which was administered using clean and dry infant feeding tube orally (PO).

Induction of DM
Distilled water was used to dissolve alloxan monohydrate and prepared a solution containing 50 mg/ml. Before the experiment, a pilot study with 100–150 mg/kg dose was conducted. Alloxan was administered by subcutaneous and intraperitoneal routes. A dose of 100 mg/kg [20] which was administered by subcutaneous route showed a fasting blood glucose (FBG) level of >150 mg/dl after overnight fasting, with lowest lethality after 21 days of DM induction. At this dose of alloxan, there was extensive pancreatic islet cells damage visualized by histopathological examination.

Hyperglycemia was induced in rats which were in fasting from overnight, by a single dose (100 mg/kg) of alloxan monohydrate in subcutaneous route. To prevent hypoglycemia during the phase of induction of DM, 5% dextrose solution for next 24 h was made mandatory along with access to food and water. Confirmation of successful hyperglycemia induction was done by estimation of FBG after 48 h of alloxan injection. The animals with >150 mg/dl of FBG were diagnosed to have diabetes and included in this study.

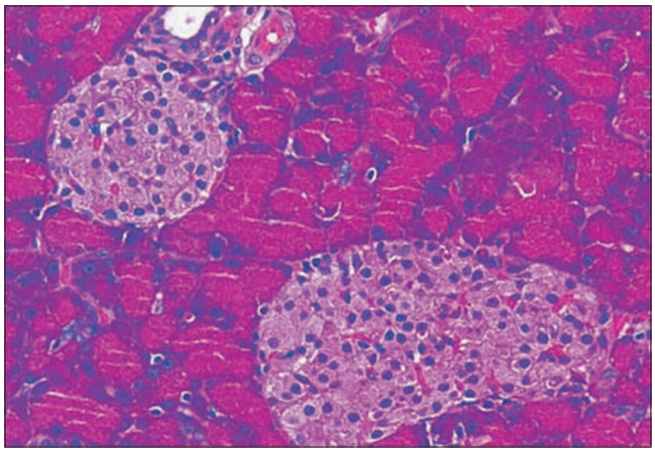

Fig. 1: Photomicrograph of pancreatic islets of normal rats

Study design
A total of 24 Wistar rats of male sex were divided into six animals in each group as four groups. They were housed in the central animal house for 6 weeks.
- Group I (n=6) normal rats. Received 1 ml distilled water
- Group II (n=6) diabetic rats. Received 1 ml distilled water
- Group III (n=6) rats with diabetes. Received silymarin 400 mg/kg PO
- Group IV (n=6) rats with diabetes. Received glibenclamide 0.5 mg/kg PO.

Group I and Group II received normal diet only with 1 ml distilled water. Group III and Group IV received silymarin (400 mg/kg PO) and glibenclamide (0.5 mg/kg PO), respectively, for 3 weeks after DM induction. On day 1, end of day 7, 14, and 21 days of DM induction, FBG levels were estimated in all four groups using glucometer. Blood samples were collected by tail snipping method [21]. At the 21st day of experiment, blood samples were retrieved by retro-orbital puncture method after intramuscular injection of ketamine [22] from all the 24 rats for biochemical analysis.

The animals were sacrificed by dislocating the cervical bone, and the diffuse pancreas from all the groups of rats was dissected out. They were processed for histopathological and biochemical analysis.

Sample preparation

Serum separation for biochemical analysis

The whole blood was collected from rats of each group in sterile, covered test tubes and labeled with anticoagulant ethylenediaminetetraacetic acid. HbA1c in whole blood was reported to be stable for a week at 2–8°C. After collection, the whole blood was allowed to clot undisturbed for 15–30 min. The clot removed by centrifuging at 1000–2000×g for 10 min in a centrifuge. The supernatant serum is obtained for biochemical analyses.

Tissue homogenate preparation for biochemical analysis

Pancreatic tissues from rats of all groups were sliced into pieces, homogenized in an appropriate buffer and kept in cold condition (pH-7.0) to 20% homogenate (W/V). The centrifugation of homogenates was done at 1000 rpm for 10 min at 0°C in cold centrifuge. The supernatant was separated, and it was invariably used for various biochemical analysis of values.

Tissue preparation for light microscopic study

For histopathological analysis, the rat pancreatic tissues were perfused with 10% formalin. The pancreas was excised from the abdominal cavity immediately after animal sacrifice and fixed in 10% neutral formalin, which was dehydrated in graded alcohol (80–100%), processed and cleaned in xylene and finally embedded in liquid paraffin. Then, the pancreas was sliced into 3–5 µm pieces with the help of rotary microtone, then deparaffinized in xylene solution passed through alcohol in various grades and finally hematoxylin and eosin-stained for histopathological assessment. The specimen's evaluation was carried out with light microscope. All histopathological changes were noted.

Statistical analysis
Values of biochemical analysis were expressed as means±standard deviation, for six rats in all four groups. The data obtained in various biochemical evaluations were analyzed by Duncan's multiple range test, by SPSS (version 1.6) software. Values not sharing a common superscript differ significantly at $p<0.05$.

RESULTS

Effect of silymarin on serum glucose levels

The mean fasting serum glucose levels of rats treated with silymarin 400 mg/kg after induction of DM, measured every week, for 3 weeks are shown in Table 1. The glucose values were compared to values obtained for diabetic control rats and glibenclamide treated rats. In all rats induced diabetes with alloxan, the FBG levels remained at high levels only for 3 weeks after induction of DM. Rats treated with silymarin (400 mg/kg) (Group III) showed a significant reduction in blood glucose levels each week ($p<0.05$), among which the maximum hypoglycemic effect was seen on 21st day after DM induction. The reduction in blood glucose level was higher in the rats treated with silymarin (400 mg/kg) on days 7, 14, and 21 which was significant reduction. In glibenclamide treated rats, blood glucose levels were lower on days 7, 14, and 21 which was significant compared to silymarin treated rats (Table 1).

Effect of silymarin on lipid peroxides (LPO) and antioxidants in pancreas

The level of LPO which is an index of lipid peroxidation was significantly increased in rats treated with alloxan when compared to normal rats (Group I). In Group III which includes diabetic rats treated with silymarin (400 mg/kg) showed significantly reduced ($p<0.05$) LPO compared to Group II diabetic rats which were left untreated and glibenclamide treated rats. Glibenclamide treated rats also reduced LPO significantly ($p<0.05$) compared to diabetic rats. The levels of superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT) were also increased significantly in rats treated with silymarin (400 mg/kg) after induction of diabetes, compared to untreated diabetic rats. The antioxidant levels were significantly higher in glibenclamide (0.5 mg/kg) treated rats compared to diabetic rats and silymarin (400 mg/kg) treated rats (Table 2).

Effect of silymarin on HbA1c

From Table 3, it was concluded that at the end of 21 days after DM induction, the HbA1c was higher in a significant manner in alloxan treated rats with DM ($p<0.05$), compared to normal rats in Group I. Diabetic rats (Group III) treated with silymarin (400 mg/kg) showed significant reduction in HbA1c, when compared to Group II diabetic rats. The hypoglycemic effect of silymarin 400 mg/kg on HbA1c was more significant than the effect of glibenclamide (0.5 mg/kg) (Table 3).

Histopathology of rat pancreas

Normal architecture with a compact arrangement of beta cells and non-beta cells. Red blood cells are visible in the vicinity (Fig. 1).

Pancreatic tissue shows atrophied and vacuolated beta cells with extensive necrosis of the islets showing starfish appearance characterised with necrotic cells, pyknotic nuclei and acidophilic cytoplasm with disruption of normal endocrine architecture (Group II) H and E × 640 (Fig. 2).

Pancreatic section shows significant cellular and architectural restoration, mild necrosis degeneration and vacuolation, and normal endocrine and exocrine pancreas (Group III) H and E × 400 (Fig. 3).

Pancreatic section showing significant cellular and architectural restoration, regeneration of islets seen, mild necrosis, degeneration and vacuolation, and normal endocrine and exocrine pancreas (Group IV) H and E × 640 (Fig. 4).

DISCUSSION

DM the most common non-communicable diseases which had become the 5th leading cause of death all over the world and its rising prevalence in an alarming rate had made India as the leading country with the highest number of diabetic subjects. Every 6 s [23], one person dies from diabetes. These statistics illustrate the importance of identifying the onset of DM and preventing their complications and thus reducing the burden on the community and the nation as a whole.

Numerous animal models and pre-clinical trials were developed for understanding the pathophysiology of DM. Chemical induction of DM by alloxan monohydrate [24] is the most reliable and potent methods which are also the easily reproducible methods among all older methods, as the rodents are more sensitive to the hyperglycemic effects of alloxan [24]. It is a well-known diabetogenic agent, used to induce Type I diabetes in animals. Rodents are the sensitive species to the

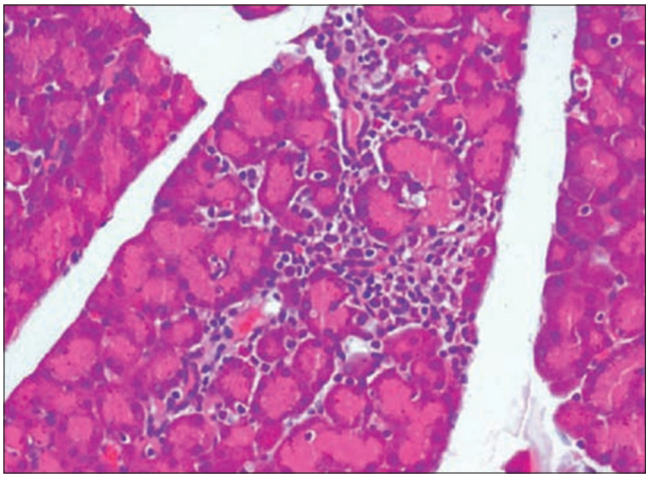

Fig. 2: Photomicrograph of pancreatic islets of diabetic rats

Table 1: Effect of silymarin on blood glucose levels in diabetic rats

Groups	Day 1 (mg/dl)	Day 7 (mg/dl)	Day 14 (mg/dl)	Day 21 (mg/dl)
Normal control	76.00±3.35^{d}	76.00±3.35^{d}	76.00±3.35^{d}	76.00±3.35^{d}
Diabetic control	202.33±2.58^{b}	184.83±4.83^{a}	201.17±9.52^{a}	206.00±10.95^{a}
Diabetes+silymarin (400 mg/kg)	208.33±11.37^{a}	171.17±2.99^{b}	143.83±6.21^{b}	118.67±3.01^{b}
Diabetes+Glibenclamide (0.5 mg/kg)	199.67±5.13^{c}	155.33±3.44^{c}	131.17±6.14^{c}	108.83±2.04^{c}

Values are expressed as mean±standard deviation for six rats in each group. Values not sharing a common superscript differ significantly at p≤0.05 (Dunnett's test)

Table 2: Effect of silymarin on lipid peroxides and antioxidants in the pancreas of diabetic rats

Groups	Superoxide dismutase (unit/mg protein)	Glutathione (µg/mg protein)	Catalase (µmol/mg protein)	Lipid peroxides (mmole/100 g tissue)
Normal control	13.28±4.69^{a}	29.11±0.20^{a}	10.19±0.33^{c}	2.53±0.25^{d}
Diabetic control	4.27±0.54^{d}	7.99±0.67^{d}	2.62±0.53^{d}	12.18±0.09^{a}
Diabetes+silymarin (400 mg/kg)	11.35±0.39^{c}	28.44±0.92^{c}	10.40±0.37^{b}	3.47±0.77^{c}
Diabetes+glibenclamide (0.5 mg/kg)	11.83±0.22^{b}	29.04±1.09^{b}	10.69±0.31^{a}	7.28±0.21^{b}

Values are expressed as mean±standard deviation for six rats in each group. Values not sharing a common superscript differ significantly at ≤0.05 (Dunnett's test)

Table 3: Effect of silymarin on HbA1c in diabetic rats

Groups	HbA1c % (glycosylatedhemoglobin)
Normal control	5.99±0.35[d]
Diabetic control	11.78±0.33[a]
Diabetes+silymarin (400 mg/kg)	7.44±0.39[c]
Diabetes+glibenclamide (0.5 mg/kg)	7.61±0.37[b]

Values are expressed as mean±standard deviation for six rats in each group. Values not sharing a common superscript differ significantly at P≤0.05. (Dunnett's test). HbA1c: Glycosylated hemoglobin

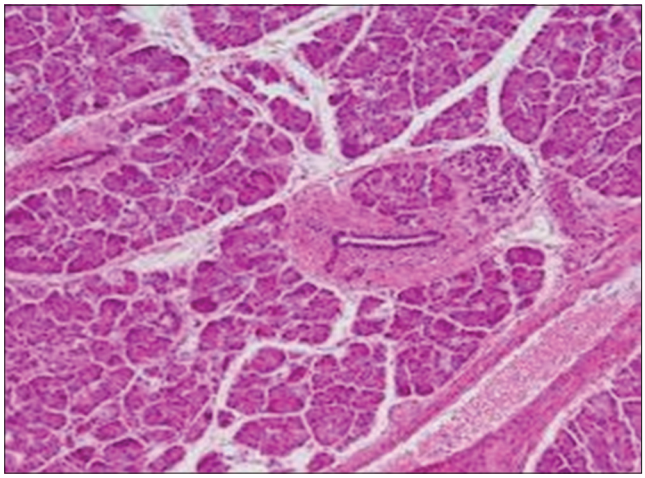

Fig. 3: Photomicrograph of pancreatic islets of diabetic rats treated with silymarin 400 mg/kg

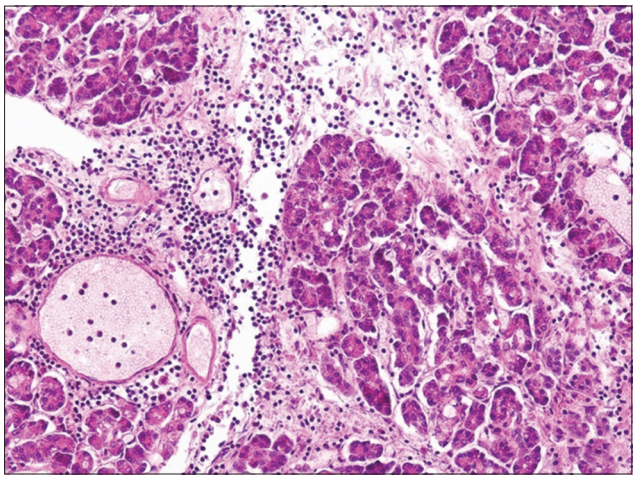

Fig. 4: Photomicrograph of pancreatic islets of diabetic rats treated with glibenclamide (0.5 mg/kg)

hyperglycemic effect of alloxan. Hence, in the present study induction of DM in male Wistar rats was done by injecting alloxan monohydrate. As the potency of the drug is very much lower in animals on feed than in overnight fasted animals [20], the animals were made to fast overnight before alloxan monohydrate injection. Alloxan selectively accumulates in beta cells through uptake through glucose transporter 2 and cause selective necrosis of beta cells within 24–48 h after the administration. In beta cells of the pancreas, the alloxan is reduced to dialuric acid in the presence of reducing agents like reduced GSH. Reoxidization of dialuric acid back to alloxan establishes a redox cycle for the generation of ROS and superoxide radicals. This causes beta-cell necrosis.

In the present study, alloxan treated diabetic rats showed marked rise in LPO and decrease in antioxidants in beta cells. The reduction in antioxidants is due to increased utilization of the above to counteract the increased formation of LPO. The damage to beta cells by alloxan was evidenced by significant rise in FBG levels (Table 1) and HbA1c (Table 3) in alloxan treated rats. The FBG levels remained significantly high on days 7, 14, and 21 after induction of DM in the study.

Silymarin is a mixture of naturally occurring flavonoid antioxidants [25]and has been shown to have other interesting activities such as cancer protective, cardioprotective, nephroprotective, neuroprotective, skin protective, and cytoprotective activities. Soto *et al*. [26] had reported that silymarin increases the pancreatic levels of free radical quenching antioxidants such as SOD, GSH, and CAT in alloxan-induced diabetic rats. Al-Enazi [27] had reported that a combination, rutin with silymarin had more ameliorative effect on oxidative stress in streptozotocin (STZ)-induced diabetic rats. Since herbal medicines are rich in flavonoids, in recent years they are gaining more importance as a major source of hypoglycemic agents [28]. Flavonoids and polyphenols have been reported to reduce various complications associated with diabetes in liver, kidneys, and blood vessels through mechanisms such as inhibition of aldose reductase, advanced glycation end-products inhibition, and the decrease of oxidative stress [29].

In the present study, hyperglycemic rats treated with silymarin showed significant reduction in LPO and increase in antioxidants and significant decrease in blood glucose levels on all the 3 weeks of the study. The HbA1c was also significantly reduced. The antidiabetic activity of silymarin could be because of its powerful antioxidant property. The cytoprotective activity of silymarin was confirmed by the histopathological examination of beta cells which show reduced beta-cell necrosis on silymarin treatment in diabetic rats and signs of regeneration of beta cells visualized in the image (Fig. 3). Hence, in the present study, alloxan treated rats showed extensive necrosis of beta cells (Fig. 2) silymarin treated diabetic rats (at 400 mg/kg) not only showed significant restoration of beta cells but also regeneration of islet cells (Fig. 3) which shows its extensive cytoprotective activity compared to glibenclamide (Fig. 4) used in the diabetes management.

CONCLUSION

The present study offers conclusive evidence that silymarin has effective antidiabetic activity and also pancreatoprotective effect against hyperglycemia-induced oxidative stress in animals. Its safety profile, easy availability, and low cost are added advantages. Hence, silymarin can be added as adjuvant therapy for controlling the blood sugar and also to prevent or slow the progression of hyperglycemia-induced microvascular and macrovascular complications. Further, studies are mandatory to conclude the role of silymarin on protein kinases, pro-angiogenic molecules and transforming growth factor-β, etc., which contribute to the complications of DM.

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AUTHORS' CONTRIBUTIONS

Uma Narayanamurthy contributed to the study design, materials and methods, animal experimentation, results in validation and discussion. Sylvia Santhakumari A contributed to animal experimentation and discussion. Nirmala P contributed to resource facilities mobilization from the university.

CONFLICTS OF INTEREST

Nil.

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