Neuroprotective and hepatoprotective effect of whole red rice forms against oxidative stress in streptozotocin induced diabetic rats.

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Received 15 May 2018; Revised 09 April 2019

Whole red rice (Oryza sativa L.), consumed as staple food in many countries worldwide, is rich in phytochemicals, mainly antioxidants having health potency. The present study evaluated the neuro- and hepatoprotective efficacy of raw and parboiled whole red rice against streptozotocin (STZ) induced oxidative stress, and antioxidant metabolism both in diabetic and normal non-diabetic rats. Wistar rats were rendered diabetic by a single intraperitoneal (i.p.) injection of streptozotocin (45 mg/kg body wt.), supplemented with raw and parboiled whole red rice for six weeks. Results revealed a drastic increase in oxidative stress markers, such as lipid peroxidation, nitric oxide (NO) level, hydroperoxide (HP) level; antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), thioredoxin reductases (TR), glutathione S-transferase (GST), acetylcholinesterase (AChE) activity, and decrease in endogenous antioxidant, glutathione (GSH) in diabetic rat. On the other hand, addition of raw and parboiled whole red rice in the diet counteracted the STZ induced oxidative stress in diabetic groups and improved the neuro and hepatic antioxidant system. Both, parboiled and raw red rice forms, exercised similar impacts in diabetic as well as in normal rats suggesting improved antioxidant defence mechanism. Results have shown that the whole red rice possess antidiabetic potential with the antioxidant improving ability, and could be utilized as dietary supplements in diabetes management.

Keywords: Antioxidants, Diabetes, Dietary supplements, Hepatoprotection, Neuroprotection, Oryza sativa, Parboiled whole red rice

Diabetes is the common endocrine metabolic disorder of the modern era leading to extensive range of physiological complications as insulin deficiency, high blood glucose levels, metabolic stress and impaired antioxidant defence systems which elicit the production of reactive oxygen species (ROS)¹. Increased ROS levels and decreased antioxidant levels lead to cellular damage by initiating mitochondrial misfunctioning, DNA disruption, increased oxidative stress and activating the inflammatory pathways²,³. Consistent production of ROS due to hyperglycemia triggered the oxidative stress through the uncontrolled production of free radicals via protein glycosylation, polyol pathways and glucose auto-oxidation pathways results in the secondary diabetic complications such as cerebrovascular and peripheral vascular disease, blindness, liver cirrhosis, neuropathy stroke, etc.¹,⁴. Elevation in ROS level in diabetes may be due to increase in the production or decrease in destruction by endogenous enzymes, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase and reductase (GPx/GR). Variation in the endogenous enzymes in the tissues makes it susceptible to oxidative stress instigating diabetic complications⁵.

Diabetes leading to neurological concerns in the central nervous system (CNS) increases the risk of dementia, stroke, mood disorders and cognitive decline. The pathological changes in brain of diabetic entities have been linked with the imbalance of antioxidant system leading to oxidative stress, non-enzymatic glycation and production of glycation end products⁶. Streptozotocin (STZ) is a monofunctional nitroso urea derivative widely used for inducing diabetes in a variety of animals by degeneration and necrosis of pancreatic beta cells⁷. Consumption of

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Abbreviations: GL, Glycemic load; GI, Glycemic index; NC, Normal control; NRJ, Normal raw whole red rice flour supplemented group; NPJ, Normal parboiled whole red rice flour supplemented group; DC, diabetes control group; DRJ, Raw whole red rice flour supplemented diabetic group; DPJ, Parboiled whole red rice flour supplemented diabetic group; STZ, Streptozotocin.
whole grains compared to the consumption of refined-grain foods could result in lower glycemic responses and insulinemia\textsuperscript{8}. Studies have reported that intake of whole grain intake can act on glucose metabolism to prevent diabetes and supported the whole grain foods for diabetes prevention and treatment\textsuperscript{9}. Itagi \textit{et al.}\textsuperscript{10} have reported that consumption of whole raw and parboiled red rice results in a reduction of hyperglycemia and hyperlipidemia. The protective effect is due to the presence of antioxidants in whole red rice may contribute to the amelioration of oxidative stress.

However, studies on the role of whole grain rice on neuro and hepatic oxidative system in general and diabetes, in particular, are limited. This study intends to estimate the \textit{in vivo} antioxidative ability of different forms of whole red rice like raw and parboiled concerning neuroprotection and hepatoprotection.

Materials and Methods

Chemicals and regents

Streptozotocin, Griess reagent, xylenol orange, sodium nitrite, quercetin, 1-chloro-2,4-dinitrobenzene (CDNB), acetylthiocholine iodide ad, ethylene diamine tetra acetic acid (EDTA) were purchased from the Sigma Chemical Co., St. Louis, USA. Research Laboratories Pvt Ltd, Mumbai, India. Sodium azide, ammonium ferrous sulphate, reduced glutathione (GSH), oxidized glutathione (GSSG), \(\beta\)-nicotinamide adenine dinucleotide 2'-phosphate reduced tetra sodium salt hydrate (NADPH) and Ellman's reagent (DTNB) secured SISCO Research Laboratories Pvt Ltd, Mumbai, India.

Red rice sample preparation and processing

Paddy of a staple variety having red pigmented bran, \textit{Jyothi}, was procured from Agriculture Products Marketing Co-operative (APMC), Bandipalya, Mysore, Karnataka, India. For parboiled sample, the paddy was soaked in boiled water and stirred continuously. The mixture was left overnight to gain the 32\% moisture level\textsuperscript{11}. The paddy was spread on wire-mesh trays to drained extra water and followed by steaming in an autoclave for 20 min. The paddy moisture was retained up to 13\% by air drying.

Milling and Pulverization

Raw and parboiled paddy was processed to remove husk using the Laboratory Satake Sheller (Satake Corporation, Tokyo, Japan). The raw and parboiled red rice (rice having all the anatomical parts such as bran, germ and endosperm) were made into flour using a rice mill (Surabhi, India) followed by sieving through a mesh and were stored at 2\(^\circ\)C until use.

Induction of diabetes

\textit{In vivo} research study was carried out on young female wistar rats, collected from CSIR-CFTRI with the permission of the Institutional animal ethics committee (approval\# IAEC no.268/2013). All rats were individually housed in stainless steel cages. A balanced commercial diet and tap water \textit{ad libitum} were provided. Control group received a saline solution by intraperitoneal injection (\textit{i.p.}). The animals were made diabetic by a single \textit{i.p.} injection of streptozotocin (45 mg/kg body wt.). Animals with blood glucose concentration above 200 mg/dL were separated into three groups and another three groups of non-diabetic animals were also included. The groups of diabetic and normal animals were given different diets as follows: Group I, Normal Control (NC); Group II, raw whole red rice flour supplemented (NRJ); Group III, parboiled whole red rice flour supplemented (NPJ); Group IV, STZ induced diabetes (DC); Group V, diabetic rats supplemented with raw whole red rice flour (DRJ); and Group VI, diabetic rats supplemented with parboiled whole red rice flour (DPJ). American Institute of nutrition reference (AIN-76) semi-purified diets were prepared by mixing ingredients in a mechanical mixer.

Normal control basal AIN-76 diet consisted of: Corn starch 54\%, casein 21\%, cane sugar 10\%, refined peanut oil 10\%, Bernhart-Tommarelli modified NRC salt 4\%, and NRC vitaminized starch 1\%. [NRC, National Research Council, adopted from American institute of nutrition reference diet (AIN 76A) with modification of source of carbohydrate]

Whole red rice diets were prepared by incorporating raw whole red rice powder 62\% in the place of corn starch in normal diet. Corn starch 0\%, Raw whole red rice flour 62\%, casein 21\%, cane sugar 10\%, refined peanut oil 10\%, Bernhart-Tommarelli modified NRC salt 4\%, and NRC vitaminized starch 1\%. Par-boiled whole red rice diets were prepared by incorporating par-boiled whole red rice powder 62\% in the place of corn starch in normal diet. Corn starch 0\%, parboiled whole red rice flour 62\% and casein 21\%, cane sugar 10\%, refined peanut oil 10\%, Bernhart-Tommarelli modified NRC salt 4\%, and NRC vitaminized starch 1\%.

Tissue sample collection

At the end of the experiment, i.e., six weeks, the rats were sacrificed under mild ether anesthesia.
During which blood samples were collected by puncturing the heart. Liver and brain samples were collected and stored at −80°C until use and processed for biochemical measurements.

**Determination of oxidative markers and antioxidative enzyme activities in brain and liver**

**Preparation of tissue samples**

The frozen whole brain, liver tissue samples were homogenized (1:10, w/v) in 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and centrifuged for 20 min. The supernatant was collected for the biochemical estimations. Protein concentration was determined by the Lowry et al. 12.

**Thiobarbituric acid reactive substances (TBARS)**

The concentration of thiobarbituric acid reactive substances (TBARS) was determined by the previously described method by Fraga et al. 13 in tissue homogenates.

**Hydroperoxides (HP) levels**

HP level was determined based on oxidation of xylenol orange mediated by ferrous ions 14. An aliquot of tissue supernatant was treated with FOX1 reagent. The reaction mixture was incubated at 37°C for 30 min. The colour developed was read at 560 nm (ε−2.2*10⁵/M/cm) and expressed as nmoles hydroperoxides/mg protein.

**Nitrite estimation**

An aliquot of sample added with of Griess reagent and incubated for 10 min of incubation at 37°C 15. The absorbance at 540 nm was determined, and nitrite concentrations were calculated from the sodium nitrite standard curve.

**Reduced Glutathione (GSH)**

GSH was determined by using the Ellman GC method 16. 1.0 mL of supernatant was treated with 0.5 mL of Ellman's reagent and 3.0 mL of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm and expressed as μmoles of GSH oxidized/min/mg protein.

**Superoxide dismutase (SOD) activity**

SOD activity was measured by monitoring the inhibition of quercetin auto-oxidation and monitored at 406 nm for 3 min 17. The ability of the sample to inhibit quercetin oxidation by 50% is defined as one SI unit of the enzyme and activity expressed as units/mg protein.

**Catalase (CAT) activity**

CAT activity was estimated by adding an aliquot to phosphate buffer (pH 7.4, containing hydrogen peroxide (H₂O₂−10 mM). H₂O₂ decomposition was monitored at 240 nm for 1min and activity expressed as nmol substrate/ min/mg protein (ε – 44.2/mM/cm) 18.

**Glutathione peroxidase (GPx) activity**

GPx activity was measured by the Paglia and Valentine method 19. The enzymatic reaction contained NADPH, GSH, and sodium azide. GPx activity was initiated by the addition of hydrogen peroxide (H₂O₂), and the change in absorbance at 340 nm for 5 min was monitored. Activity was given in units/g protein.

**Glutathione reductase (GR) activity**

The activity of glutathione reductase was measured by the method described as in a 1 mL reagent containing 50 mM phosphate buffer pH 7.0, 1 mM EDTA, 10 mM GSSG, and 0.1 mM NADPH. The activity expressed as nmol NADPH oxidized/min/mg protein (ε – 6.22/mM/cm) 20.

**Thioredoxin reductase (TR) activity**

TR activity was measured by the Luthman & Holmgren method 21 by adding sample aliquot in reagent mixture containing 100 mM potassium phosphate buffer pH 7.0 containing 10 mM EDTA, 0.2 mM NADPH. The activity was measured by monitoring the reduction of DTNB at 412 nm and expressed as nmol substrate reduced/min/mg protein (ε – 13.6/mM/cm).

**Glutathione-S-transferase (GST) activity**

Glutathione-S-transferase (GST) activity was quantified based on the conjugation of glutathione to CDNB 22. The assay was started by adding an aliquot in reagent mixture containing potassium phosphate buffer pH 6.8 containing EDTA, 0.75 mM CDNB, 0.05 mM GSH. The change in O.D at 340 nm was recorded over 3 min explain, and the activity expressed as nmol conjugate formed/min/mg protein (ε – 9.6/mM/cm).

**Acetylcholinesterase (AChE) activity**

AChE activity was estimated according Ellmann et al. by adding of sample aliquot to reaction mixture containing acetylthiocholine iodide (1.95 mM), phosphate buffer (0.1 M, pH 8.0) and DTNB (2.5 mM) 23. The change in absorbance was monitored at 412 nm for 3 min. and the enzyme activity was expressed as μmoles substrate hydrolyzed/min/mg of protein.

**Statistical analysis**

Values are the average ± standard deviation of 6 analyses. Values were subjected to Student t-test to study at the significance level P <0.05 by Snedecor & Cochran 24.
Results

Effect of supplementation on oxidative markers in normal and diabetes groups

Supplementation with whole raw red rice did not show any change in MDA level in brain and liver (Fig. 1A), whereas whole parboiled red rice showed a significant decrease in MDA level in the brain of the normal rat. Elevation in MDA levels was observed in both liver (99%) and brain (61%) of Control diabetic rats compared to normal control rats. Dietary supplementation to diabetic group with a raw and parboiled diminished brain (7 and 25%) and hepatic (26.5 and 26%) MDA levels, respectively when compared to the diabetic control. In this study, nitrite levels were significantly increased in the brain and liver of diabetic rats compared to the normal control (Fig. 1B). Supplementation to diabetic groups significantly decreased the NO level in both brains (37 and 45%) and liver (44 and 55%). Hydroperoxides level (HP) was observed to be significantly elevated in brain of diabetic Control compared to normal control (Fig. 3D). Red rice forms supplementation to diabetic group showed a significant reduction in DRJ (27%), DPJ (23%), in the hydroperoxide level.

Effect on the endogenous antioxidant, glutathione (GSH) levels in normal supplemented and STZ induced diabetes

The change in the level of GSH (Fig. 1C) was found elevated in the normal supplemented group in the liver and found no change in brain GSH level as compared to the normal group. Diabetic brain and liver showed 81% and 105% significant decrease in GSH levels compared to control rats. Supplementation with whole rice to diabetic group significantly increased the GSH level DRJ (52%), DPJ (67%) in brain and DRJ (119%), DPJ (76%) in liver, respectively.

Effect on the antioxidant enzymes in normal supplemented and STZ induced Diabetes:

In normal control group, raw red rice supplementation showed reduction in SOD, and CAT TRx activity by 12, 13 and 27%, respectively, whereas no effects were observed on GPx, GR (Fig. 2 A-D) and GST (Fig. 3B) activity in brain. Control parboiled red rice supplemented group resulted the reduction in brain SOD, CAT, GR and GST activity by10, 20, 14 and 27%, respectively whereas there were no effects on GPx and TRx (Fig. 3A) activity. Supplementation of raw and parboiled red rice showed significant increase in acetylcholinesterase (AChE) activity (Fig. 3C) in the brain of control rats. Diabetic control group resulted in the augmentation of antioxidant enzymes and AChE activity. Whole rice supplementation significantly diminished the SOD activity in the brain of DRJ(47%) and DPJ (45%), CAT in DRJ (35%) and DPJ (37%), GPx activity in DRJ (45%) and DPJ (50%), GR activity in DRJ (25%) and DPJ (49.5%), GST activity in DRJ (10%) and DPJ (10%), TR activity in DRJ (46%) and DNPJ (45.5%) and AChE activity in DRJ (21%) and DPJ (15%). In the liver, whole rice supplementation significantly diminished
the antioxidant enzymes activity and came down to normal. Whole rice supplementation significantly diminished the liver SOD activity by DRJ (46%) and DPJ (37%), CAT in DRJ (24%) and DPJ (20%), GPx activity in DRJ (45%) and DPJ (50%), GR activity in DRJ (64%) and DPJ (71%).

**Discussion**

Oxidative stress has been proposed as a primary culprit in the diabetes pathophysiology. Several *in vivo* and *in vitro* studies have demonstrated increased, or uncontrolled oxidative activity is probably associated with the diabetes-linked antioxidant defense enzymes system, such as glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT) and non-enzymatic scavengers like reduced glutathione. Diabetes-induced hyperglycemia causes oxidative stress in various brain regions, recognized to increase the extent of neurological disorders due to the inhibition of enzymatic activities connected to neurotransmission. Whole rice forms possess the beneficial components that may offer important health benefits, including the beneficial effect on oxidative stress reduction and...
other metabolic activities. The schematic depiction of the present study is represented in Fig. 4.

Study elucidates that supplementation with whole red rice forms did not affect the MDA level in normal controls, which shown oxidative tissue system was not affected except in the brain of the parboiled supplemented group. Studies have shown that STZ-induced diabetic rats had significantly increased MDA concentration in the liver and Brain. Elevation in MDA indicated that there was a higher production of ROS that increased the rate of lipid peroxidation. According to Yoshikawa et al.\textsuperscript{26}, lipid peroxidation can result in several mechanisms similar to impairment of the function of membrane ion-motive ATPases the glucose transporters, generation of gene products such as nitric oxide and glutamate transporters that cause cellular damage. Nitric oxide (NO) may react with ROS like the superoxide radical and result in the production of the highly reactive oxidant species peroxynitrite, leading to further destructive oxidative and nitrostatic stress that may contribute to secondary complications. Elevated NO level suggested as one of the pathophysiological mechanisms of STZ-induced diabetes. The oxidative damage to the brain and liver may be a result of excess production of NO by forming peroxynitrite.
with superoxide. Results were in agreement with previous studies which demonstrating an increase of lipid peroxidation, NO level, increased activity and expression of liver nitric oxide synthase in liver and brain in experimental diabetes\textsuperscript{27,28}. Whole rice supplementation significantly protects against the formation of lipid peroxides and results in significantly decreased MDA, NO and HP levels in diabetic rats. Our results revealed that the protective role of whole rice could be due to the antioxidative effect of the natural phytonutrients found in whole red rice, such as polyphenols, oryzanol and vitamin E. Esa et al.\textsuperscript{28} also reported that brown rice and germinated brown rice have protective effects against stress-induced liver injury via antioxidant activities, as shown by both \textit{in vivo} and \textit{in vitro} assays. The increased SOD, CAT, GPx, GR and GST activity in diabetic compared to control could be due to its induction by increased production of superoxide, and H$_2$O$_2$ also reported to act as an inducer of tissue SOD and may be another sign for the increased oxidative stress in the liver and brain tissue. The increase in CAT activity may indicate a high degree of oxidative stress resulting in the increased endogenous H$_2$O$_2$. The results are in agreement with earlier published findings wherein increased in enzymes activity in diabetic rats\textsuperscript{29-33}. A probable mechanism explaining these changes may be related to the increased glucose concentration in diabetes. Endogenous thiol antioxidant, thioredoxin (TRx) plays a vital role in cell function by controlling cellular redox status and protection against oxidative stress through its antioxidant effects, and by protein-protein interactions with critical signaling molecules. Present study observed a significant increase in diabetic rats in TRx activity and as in an earlier study which reported that diabetes increased TRX-1 and TXNip mRNA levels in the brain\textsuperscript{34}. The present study showed a significant decrease in the endogenous antioxidant, GSH level in the brain and liver of diabetic rats. Our data are consistent with described a decrease in GSH levels in the diabetic rats\textsuperscript{35}. Supplementation with raw and parboiled attenuates the GSH level in diabetic groups compared to the diabetic control group, and this may be because of exogenous antioxidants obtained from whole red rice. AChE enzyme is crucial in maintaining the
nervous system normal function since it terminates
the action of ACh released into the synapse. Increased
in brain AChE activity is observed in diabetic group
compared to control in our study, supported by other
studies also showed increased AChE activity, as this
enzyme hydrolyzes acetylcholine in the brain and
marks in cognitive decline. Reports have demonstrated
elevated AChE activity in the brain after STZ
administration.\textsuperscript{36} Supplementation of diabetic group
with aqueous extract of vitexnegundo (AEVN) also decreases
acetylcholinesterase activity which could be due to
flavonoids content of the extract.\textsuperscript{37}

These beneficial effects may be because of the
antioxidant component in red rice, such as
polyphenols, oryzanol, tocopherols, tocotrienols, and
dietary fibers. Research reported the reduction of
liver TBARS and increasing liver glutathione and
glutathione peroxidase by a mixture of brown and
black rice that was more effective in reducing than
was white rice alone.\textsuperscript{38} Supplementation of coloured rice
significantly decreased liver reactive oxygen
species and aortic MDA, while significantly increased
the total antioxidant capacity, compared to those
fed white rice.\textsuperscript{39} Reports show that parboiled rice that
has higher levels of total polyphenols than white rice,
has increased glutathione peroxidase (GP\textsubscript{X}) activity
and glutathione (GSH) content, possibly conferring
protection against oxidative stress to renal tissue in
diabetic rats.\textsuperscript{40} Polyphenols are highly antioxidative
in nature and may offer protection in neurological
diseases. Reports show protection of neurons
against a variety of experimental neurodegenerative
conditions cognitive deficits associated with
diabetes.\textsuperscript{41} Polyphenols and flavonoids are known
to prevent LDL oxidation in in vitro conditions.\textsuperscript{42}
Evidence showed that major rice component, gamma-
Oryzanol can stimulate glucose uptake via the
regulation of mTORC1 signaling and a significant
constituent in obesity associated metabolic disorders
for the health benefits of brown rice.\textsuperscript{43} Studies have
demonstrated that vitamin E improves the oxidative
stress parameters in both animals and humans.\textsuperscript{44,45}
Vitamin E supplementation improves the antioxidative
defense system through an increased activity superoxide
dismutase (SOD) and glutathione peroxidase and
increased vitamin E content in the brain of the
diabetes-induced experimental rats. Studies have
shown that the protective effect of alpha-tocopherol
against STZ induce oxidative stress and is recognized
to neutralize free radicals, confiscate the superoxide
anions generated by oxidative stress.\textsuperscript{45}

In addition to the beneficial effect in diabetes,
the most noteworthy result in this study was that the
supplementation with whole raw red rice in normal
control compared to refined diet is resulting reduction
in oxidative stress markers like MDA, NO, HP as well as
antioxidant enzyme machinery with respect to
SOD, CAT, TR activity and absence of change in
GSH metabolism. In the case of whole parboiled red
rice, it has shown a reduction in oxidative markers
like MDA, NO, HP as well as antioxidant enzyme
machinery concerning SOD, GR, GST, and higher
CAT activity.

Conclusion
Effect of supplementation of whole red rice forms
against oxidative stress in the brain and liver of
normal and STZ induced diabetic rats were carried
out. Reduction in oxidative stress markers, as well as
improvement in endogenous antioxidant and
antioxidant enzymes activity in both brain and liver of
the diabetic group, was observed on supplementation
with whole red rice. In addition, whole red rice forms
has also demonstrated beneficial effect against
oxidative stress and improving the antioxidant system
in normal rats. This beneficial effect may be because of
phytochemicals present in whole red rice forms.
Based on the study, the consumption of whole red rice
forms can be recommended for prevention of
secondary complication associated with diabetes.

Acknowledgment
Author Saroj Yadav was supported by the DST-
INSPIRE Fellowship for Junior Research Fellow.

Conflict of Interest
The authors declare that there are no conflict of
interests.

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