IJPSR (2017), Volume 8, Issue 9 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 02 February, 2017; received in revised form, 12 August, 2017; accepted, 23 August, 2017; published 01 September, 2017

EFFECT OF COMBINED MIXTURE OF FOUR MINOR MILLETS ON BLOOD GLUCOSE, SERUM LIPIDS AND OXIDATIVE STRESS IN OVARIECTOMISED WISTAR ALBINO RATS AN ANIMAL MODEL FOR MENOPAUSAL COMPLICATIONS

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Keywords:

Menopause, Minor millets
Bilaterally ovariectomised rats,

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ABSTRACT: Diabetes mellitus, coronary artery disease and oxidative stress are some of the important complications encountered by women during menopause due to decrease in estrogen levels. Food intake has a strong impact on menopausal women and a well balanced diet is important for good health and to combat some of the complications of menopause to certain extent. Minor millets has much health benefits and help to overcome non-communicable diseases like diabetes mellitus, obesity, hyperlipidemia, cardio-vascular diseases, *etc.* It is rich in micronutrients, especially vitamin B and minerals, Phytochemicals and dietary fiber. The phenolic compounds in minor millets also helps in the prevention of chronic diseases like Cancer. Minor millets like *Paspalum scrobiculatum* L., *Panicum sumatrense* Rothex Roem. and *Schult, Eichinochola frumentacea* Link and *setaria italic* (L.) *P. Beauv.* were chosen for the study. The bilaterally ovariectomized rat is an animal model for the studies related to menopause. The powdered minor millets were mixed with rat pellet and fed to the ovariectomised rats. After three months of the study, the blood was collected and analysed for Blood Glucose, Serum Lipid profile, TBARS, Gammaglutamyltransferase. There was a significant ($P< 0.05$) decrease in the serum glucose,HbA1c, LDL cholesterol, Triglycerides, TBARS and GGT and improvement in serum HDL cholesterol in the Minor millets fed group compared to the control(SHAM operated). Thus Minor millets chosen for the study has the ability to keep blood glucose level and serum lipid level in control and have the ability to reduce the oxidative stress parameters.

INTRODUCTION: Menopause is the event in which the ovarian follicles get exhausted, as a result there is a decrease in the production of estradiol and other hormones [1]. Further, the much reduced estrogen during menopause increases the level of free fatty acid, which makes postmenopausal women easily prone to the metabolic syndrome and insulin resistance, which are the risk factors for cardiovascular disease [2].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.8(9).3822-29

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.8 (9).3822-29

Metabolic Syndrome and Cardio Vascular Disease which are more common in older women with notable increase in individual risk factors in the postmenopausal phase [4].

Women have a more tough phase of old age than men because of the dominant effects of hormonal changes caused by menopause. However, the public health care system does not concentrate the special health needs of older women. There has been enormous research on menopause in the West but in India only a few institutes have recognised the importance of research on the subject [5]. Nutrition is a basic need for human and an important factor to lead a healthy life. From the very early stages of life, a proper diet is essential

for proper growth, development and to remain active [6]. Symptoms of menopause may be decreased by altering their nutritional status, which is one of the important environmental factors to lead a healthy life during menopause [7].

At different stages of life, the human body changes and it requires unique eating habits to sustain normal physiological functions. The increasing urbanisation, westernization and mechanization in most countries has led to a sedentary lifestyle and a diet having high energy-dense and high fat foods [8, 9].

Small millets, grown as a complement to existing crops, could offer an answer to the malnutrition and non-communicable diseases. They have good nutritional properties, including high micronutrients, low glycemic index, dietary fiber content[10], low digestibility, low carbohydrate content and water soluble gum content (b-glucan) which improves glucose metabolism. Thus they release sugar slowly in the blood and also slows the glucose absorption [11]. Thus they exhibit hypoglycemic and hypolipidemic effects [12]. The bilaterally ovariectomized rat model is the most popular choice of animal model for the study related to menopause, as it has been proven to represent some of the most important clinical features like postmenopausal bone loss [13], cardiovascular dysfunction [14], metabolic changes [15] and oxidative stress (OS) [16].

Though there are more number of pharmacological studies carried out worldwide on minor millets, no research work have been carried out in ovariectomized rats, an animal model of menopausal complications. This research work was done to study the potential effect of four minor millets when consumed as combined mixture on clinical and biochemical and clinical parameters of the metabolic syndrome in experimental menopause-induced rat model. Thus the aim of current study was to determine the metabolic impact of selected four minor millets when consumed as feed by ovarictomized rats.

MATERIALS AND METHODS:

Plant Materials: Minor millets (*Paspalum scrobiculatum* L., *Panicum sumatrense Rothex Roem. & Schult, Eichinochola frumentacea* Link and *setaria italic* (L.) *P. Beauv.)* were purchased from a local shop in Erode, Tamil Nadu, India and was identified and authenticated by Dr. K. Althaf Ahamed Kabeer, Scientist -D, Botanical survey of India, Southern regional centre, Coimbatore, Tamil Nadu, India. All the minor millets were taken in equal proportion, subjected to cleaning with water for 10 minutes and then they are rinsed with distilled water and then air-dried overnight in an oven at 40 °C. The Minor millets were ground to coarse powder and the powder was mixed with water to form dough which was rolled and cut as the size of rat chow pellet. Then the pellets were subjected to baking overnight in an oven at 40 °C to dry out most of the moisture content so that it can be stored until the end of the study. The pellet was packed in a tight plastic container to keep it fresh and free from humidity [17].

Phytochemical analysis of the aqueous extracts of the selected minor millets and its mixture revealed the presence of phenolic compounds, phytosterols, carotenoids, flavanoids, calcium, iron, steroids, alkaloids, carbohydrates and glycosides in extract. It was also found to have antioxidant activity.

Animals: Twelve female wistar albino rats weighing about 170 - 200 g in the age group of about 90 days were used and acclimatized to the experimental room at temperature 23±2°C, controlled humidity conditions (50–55%) and 12-h-light/12-h-dark cycle. Animals were caged with a maximum of three animals each in a polypropylene cage and were fed with standard food pellets and water *ad libitum*. Ethical clearance was obtained from the Institutional Animal Ethics Committee (Proposal No. NCP/IAEC/No:07/2014-15)) for carrying out the animal study at Nandha Pharmacy College, Erode, Tamil Nadu.

Experimental design: After seven days of acclimation, the rats were ovariectomized (OVX) or sham operated. The rat were anesthetized with ketamine hydrochloride (70mg/kg i.m), the ovaries were removed ventrally. Sham operation was performed in same manner but only exposing the ovaries. On sutures broad spectrum antibiotic were applied for ten days. Ten days after recovering from surgical damage, Experimental animals were divided randomly into four groups of three animals each.

Group 1 was sham operated which served as basal control. All the other groups were ovariectomized and received treatment for 11 weeks starting from the fifteenth day of ovariectomy. Group 2 received the normal rat pellet diet and served as ovariectomized control. Group 3 was orally administered with Estradiol valerate (progynova tablets - contain the active component estradiol valerate which is a naturally occurring form of the main female sex hormone, oestrogen) (2mg/animal/ day) orally for 11 weeks. Groups 4 were fed with the feed prepared from the four minor millets Body weights of all animals were measured weekly. At the end of 11 week treatment, all the rats were deprived of food. On next day, blood samples from all the groups were collected through sinus puncture. The blood samples were centrifuged at 2500 rpm for 15 minutes to separate serum and preserved (-20 °C) for analysis of blood glucose, HbA1c, Hemoglobin and lipid profile. Soon after collecting of blood, the animals were sacrificed by cervical dislocation and the heart was carefully removed, cleaned and weighed and preserved in 10% formalin solution for histopathological analysis[18, 19].

FIG 1: SAMPLES OF MINOR MILLETS USED IN THE STUDY

FIG. 2: FEED PREPARED FROM THE FOUR MINOR MILLETS

FIG. 3: STANDARD DRUG – ESTRADIOL VALERATE

FIG. 4: SHAM OPERATED WISTAR ALBINO RAT

FIG. 5: OVARIECTOMISED WISTAR ALBINO RAT

Biochemical Assays of Serum: The level of blood glucose, HbA1c and the lipid profile in serum were determined by automatic analyser (Cobas C 111) using standard methods.

Analytical Procedures: Estimation of blood glucose was done by UV Test, Enzymatic reference with hexokinase method [20, 21]. HbA1c was estimated by Eross et al method [22]. Estimation of serum cholesterol was carried out by the method of Roeschlau and Allain [23, 24]. Serum triglycerides were estimated by the method of Wahlefeld [25] and HDL cholesterol was estimated by the homogenous enzymatic colorimetric assay [26, 27]. The VLDL cholesterol was calculated using the formula, TG/5mg/dl. The serum LDL cholesterol was estimated by the homogenous enzymatic colorimetric assay, an automated method [28]. Atherogenic index was calculated by using the formula, TC-HDL-C/HDL-C [29]. Estimation of lipid peroxidation was done by Thiobarbituric acid reaction [30]. Estimation of GGT was done by Szasz [31].

Data analysis: Results were given as mean ± S.D. Data were analyzed using T test and one-way analysis of variance (ANOVA). A p-value of 0.05 or less was considered as indicator of a significant difference.

RESULTS AND DISCUSSION: The effect of minor millets on body weight, blood glucose, HbA1c and Hb are summarized in **Table 1**. At the beginning of the study the body weight, blood glucose and HbA1c of the OVX and Sham operated rats were not significantly ($P < 0.05$) different but after the start of the study OVX control group showed significant ($P < 0.05$) increase in the body weight whereas the minor millets fed group were able to keep the weight under control within three weeks of the start of the study itself.

TABLE 1: EFFECT OF SELECTED MINOR MILLETS ON BODY WEIGHT AND SERUM BIOCHEMICAL MARKERS IN OVARIECTOMISED RATS

Group	Body weight(g)		Blood Glucose(mg/dl)		HbA1c(%)		Hb (mg/dl)	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Sham operated	199±8.66	250.33±7.57	82.73±2.05	82.73±2.05	4.23±0.25	5.1±0.44	15.77±0.25	15.73±0.21
Ovx control	199±14.73	286.67±9.87	114.33±1.53	116±1.55**	7.63±0.35	7.77±0.81**	9.97±1.0	10.37±0.47**
Ovx + std. drug	194.67±5.03	213.67±18.01	115.5±0.5	91.2±0.72	7.43±0.93	5±0.2	10.47±0.6	14.5±0.5
Ovx + MM	200±2.0	203±4.73*	117.9±3.01	80.37±0.55*	7.93±0.23	4.5±0.3*	10.37±0.57	15.3±0.3*

Mean ± S.D (n = 3). **statistically significant ($P < 0.05$) from the sham-operated control group, * statistically significant ($P < 0.05$) different from the OVX control group. Ovx control -Ovariectomised control, Ovx + std. drug - Ovariectomised + standard drug control, Ovx + MM - Ovariectomised + minor millets fed

The blood glucose and glycosylated hemoglobin level showed a dramatic reduced level in minor millets fed group compared to the ovariectomised control group. The Hemoglobin level was much reduced after ovariectomy but after the beginning of the study, there was a gradual improvement in the hemoglobin level in the minor millets fed group.

Total cholesterol, LDL, TG and VLDL level in the serum of the ovariectomized control rats was found to be markedly higher than in the sham-operated controls (**Table 2** and **3**). Minor millets fed group showed significant ($P < 0.05$) reduction in total cholesterol, LDL, TG and VLDL compared to the OVX control. HDL level was significantly ($P < 0.05$) increased in the minor millets fed group.

TABLE 2: EFFECT OF SELECTED MINOR MILLETS ON SERUM LIPID PROFILE IN OVARIECTOMISED RATS

Group	Total Cholesterol (mg/dl)		HDL(mg/dl)		LDL(mg/dl)	
	Initial	Final	Initial	Final	Initial	Final
Sham operated	185±5.0	183.33±2.08	70±2.0	69.33±3.79	86±6.56	88.33±2.08
Ovx control	251±9.87	260±10**	37.67±2.52	37.67±2.51**	179±11.53	183.67±1.53**
Ovx + std drug	266.67±3.06	175.33±5.03	33.33±2.31	67.67±2.5	177±8.19	87.67±1.53
Ovx + MM	279.33±19.22	174.33±8.14*	38±2.0	71.33±1.5*	182.67±8.33	85.67±2.08*

Mean ± S.D (n = 3). **statistically significant ($P < 0.05$) from the sham-operated control group, * statistically significant ($P < 0.05$) different from the OVX control group. Ovx control -Ovariectomised control, Ovx + std. drug - Ovariectomised + standard drug control, Ovx + MM - Ovariectomised + minor millets fed.

TABLE 3: EFFECT OF SELECTED MINOR MILLETS ON SERUM LIPID PROFILE IN OVARIECTOMISED RATS

Group	TG (mg/dl)		VLDL (mg/dl)	
	Initial	Final	Initial	Final
Sham operated	158.33 ± 9.07	168.67 ± 1.15	26.53 ± 0.42	26 ± 0.2
Ovx control	262.67 ± 11.02	268.67 ± 3.2**	52.53 ± 2.2	53.73 ± 0.64**
Ovx + std drug	258.67 ± 6.03	158 ± 2.0	51.73 ± 1.21	25.73 ± 0.12
Ovx + MM	266.33 ± 5.51	156.67 ± 9.87*	51.67 ± 0.58	25.67 ± 0.5*

Effect of Minor Millets on Serum Oxidative Stress Parameters: Minor millets fed ovariectomised group showed decrease in the oxidative stress (TBARS). Present study result (**Fig. 6**) showed significant increase in blood serum TBARS in Ovariectomised control rats compared to Sham control rats. The Standard drug (Estradiol Valerate) treated and Minor millets fed group prevented the formation of reactive oxygen species and induction of lipid Peroxidation by Ovariectomised rats.

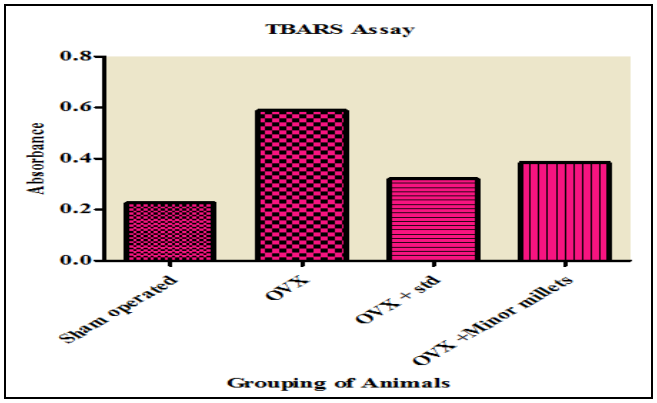

FIG. 6: EFFECT OF SELECTED MINOR MILLETS ON THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS) IN OVARIECTOMISED RATS

Minor millets fed ovariectomised group showed decrease in the Gamma Glutamyl transferase (GGT). Present study result (**Fig. 7**) showed significant increase in blood serum GGT in Ovariectomised control rats compared to Sham control rats. The Standard drug (Estradiol Valerate) treated and Minor millets fed group prevented the formation of reactive oxygen species and induction of lipid Peroxidation by Ovariectomised rats

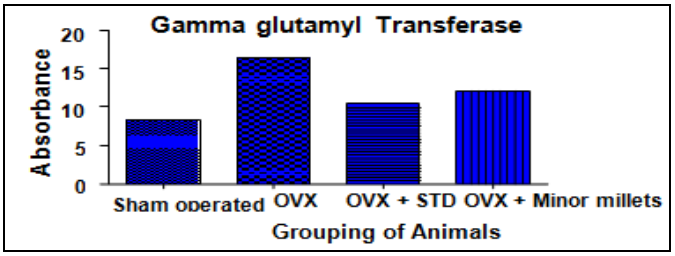

FIG. 7: EFFECT OF SELECTED MINOR MILLETS ON GAMMA GLUTAMYL TRANSFERASE (GGT) IN OVARIECTOMISED RATS

Postmenopausal status is associated with an increased risk of Diabetes mellitus, Coronary artery disease and Oxidative stress. Therefore, to prevent the above complications, there is a need to evaluate the blood glucose, lipid profile and oxidative stress parameters from the time of the menopause [32], in which Low HDL-cholesterol level, high abdominal obesity and low-grade chronic inflammation [33] are the most frequent characteristics in comparison to other metabolic components. The increased body weight in ovariectomized rats is well-documented in many studies [35]. Some previous studies in ovariectomised rats showed that estrogen deficiency significantly led to weight gain [36, 37]. The results of the present work revealed the weight control ability of the minor millets in ovariectomised rats.

Estrogen also decreases blood glucose and glycosylated hemoglobin, a marker of long-term vascular damage in diabetes [38, 39]. Hemoglobin level was found to be much reduced in ovariectomised rats which might be due to the increased formation of HbA1c. In poorly controlled diabetes, there is an increased glycosylation of a number of proteins including hemoglobin [40]. HbA1c was found to increase in patients with diabetes mellitus to approximately 16% and the amount of increase was directly proportional to the fasting blood glucose levels [41]. So, the measurement of HbA1c is a very sensitive index for glycemic control.

Total cholesterol, LDL-C and triglycerides were elevated and HDL cholesterol was decreased in post menopausal women [42]. Lipid profile and atherogenic index have been shown to be strong (significant) predictors for metabolic disturbances including Dyslipidemia, atherosclerosis, hypertension and cardiovascular diseases. Any changes in the levels of lipids make the individuals more likely to develop atherosclerotic cardiovascular diseases as well as endothelial dysfunction [44].

High density lipoprotein removes excess cholesterol from peripheral tissues and transports it to the liver, thus by cholesterol reverse transport, cholesterol homeostasis will be maintained [45, 46]. Lowering of serum lipid levels by dietary or drug therapy seems to be associated with a reduced risk of vascular disease [47].

One of the major risk factors for developing cardiovascular disease is the elevated cholesterol. Ovariectomy lead to undesirable changes in the lipid levels thus leading to atherosclerosis and coronary heart disease [48, 49]. The present work investigated whether the selected minor millets were effective in reducing the increased serum cholesterol level in ovariectomised rats. Deficiency of estrogen have an indirect effect on lipid profile [50]. Coronary heart disease is directly linked with increased LDL cholesterol and inversely linked with increased HDL cholesterol [51]. Millets have been reported to be the rich sources of dietary fibre [52], resistant starch and low glycemic response [53, 54] thus attributed to exhibit hypoglycemic and hypolipidemic effects [55, 56].

Minor millets, with their low carbohydrate content, low digestibility and water soluble gum content (b-glucan) have been reported to improve glucose metabolism. These grains release sugar slowly in the blood and also diminish the glucose absorption [57, 58]. These grains have also been demonstrated to exhibit beneficial effects on cholesterol levels, which is also due to their high dietary fibre and phytochemical content.

Elevated plasma glucose develops Reactive Oxygen Species (ROS) which leads to oxidative stress [59]. There is also a decrease in serum GGT in the minor millets fed group which is an indicative of the decreased oxidative stress. Oxidative stress leads to the pathogenesis of hypertension [60]. In another study it was found that there was increase in serum GGT with enhanced oxidative stress and reduced antioxidant defense system in the post-menopausal women which leads to the speculation that GGT could be considered an index or a marker of oxidative stress [61]. The beneficial effects of antioxidants in releaving oxidative stress and reducing elevated blood pressure is shown in various clinical studies [62]. And the antioxidants have also been proven to reduce the oxidative stress accompanied by decreased levels of plasma glucose and glycosylated hemoglobin, which is supported by the hypoglycemic effect of honey accompanied by reducing oxidative stress. Similarly, the antioxidative properties of minor millets against hyperglycemia and oxidative stress are due to rich reserves of macro nutrient, micro nutrient and phytochemicals like phenolics, tannins, phytates, *etc*.

CONCLUSION: It is concluded that Blood glucose, Lipid profile and serum oxidative stress parameters are found to be elevated in the ovariectomised Wistar albino rats, which indicates that the menopausal women are at great risk of complications like Diabetes mellitus, Hypertension ,coronary artery diseases and even depression. Minor millets may be minor in their size but superior in its nutrients. They contain many health beneficial components which have been shown to reduce many degenerative diseases of the mankind like diabetes mellitus, cardiovascular diseases, cancer, *etc*.

This age of mankind can be better termed as “diseased period” because we are suffering from innumerable diseases of which source and cause are still under dark. We are not living but dying each and every second of our life by the fear of attack of the disease. Thus the only solution to be freed from such panic is to change the food habit and consume nutrient foods like minor millets.

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How to cite this article:
Prabha KC and Selvi S: Effect of combined mixture of four minor millets on blood glucose, serum lipids and oxidative stress in ovariectomised wistar albino rats an animal model for menopausal complications. Int J Pharm Sci Res 2017; 8(9): 3822-29.doi: 10.13040/IJPSR.0975-8232.8(9).3822-29.