Soy isoflavones (Glycine max) protect free radical induced red blood cell damage and plasma protein degradation. Evidence for antioxidant activity of soy isoflavones

Sankar P[1], Zachariah Bobby[1], Sajini Elizabeth Jacob[2]

[1]Department of Biochemistry, Jawaharlal Institute of Postgraduate Medical Education and Research [JIPMER], Puducherry – 605 006, India
[2]Department of Pathology, Jawaharlal Institute of Postgraduate Medical Education and Research [JIPMER], Puducherry – 605 006, India

Abstract- Oxidative stress and red cell damage underline most pathological consequences. Isoflavones are group of compounds derived from soya beans. There is confusion in the literature regarding the antioxidant property of isoflavones. Hence, the present study was conducted to evaluate the antioxidant property of soy isoflavones and its protective role on red cells against free radicals and plasma protein degradation. Isoflavones were extracted from soya bean and free radical scavenging capacity was assessed by DPPH assay. It was found that the free radical scavenging capacity of isoflavones was 6.23 ± 1.04 µM/L equivalents of Gallic acid. To assess the antioxidant property of isoflavones, the red cells were incubated with 60µg/ml and 120µg/ml of isoflavones and were exposed to glucose at physiological and pathological concentrations for 24 hours. In another experiment, the plasma was incubated with 30µg/ml, 60µg/ml and 120µg/ml of isoflavones and were exposed to 100mM AAPH for 2 hours. After incubation, the glycated hemoglobin (HbA1c), glucose, malondialdehyde, reduced glutathione, red cell count and hemoglobin indices, plasma malondialdehyde, protein carbonyl content and protein separation were estimated. The glycated hemoglobin, RBC and plasma malondialdehyde and plasma protein carbonyl levels were significantly higher, red cell count and hemoglobin concentration were significantly reduced in red cells incubated with 50mM glucose and AAPH incubated plasma. The increase in HbA1c, RBC and plasma malondialdehyde, protein carbonyl content and decrease in the red cell count were blocked significantly when red cells and plasma were pre-incubated with isoflavones. Microscopic analysis revealed that the red cells incubated at 50mM concentration of glucose possessed morphological abnormalities. However, these abnormalities were prevented in cells when pre-incubated with isoflavones of 120µg/ml concentration, but not in 60µg/ml. Similarly, electrophoresis of plasma proteins revealed that there was significant protein degradation in AAPH treated plasma and this effect was blocked by soy isoflavones treatment. Taken together, our study suggests that the soy isoflavones protect red blood cells and plasma protein degradation from oxidative stress.

Index Terms- Soy isoflavones, Glycated hemoglobin, Lipid peroxidation, Glutathione.

I. INTRODUCTION

Red blood cells (RBCs) are unique, highly specialized and the most abundant cells in the human organism. It plays a crucial role in effective anti-oxidative systems that make them mobile free radical scavengers, providing antioxidant protection not only to themselves but also to other tissues and organs in the body [1, 2] . A number of in vitro/ in vivo studies have proved that several erythrocyte parameters are negatively affected by oxidative stress and the red cells are one of the first cells to be affected by changes in the redox status of the body [3–5] . Alterations in red blood cells are widely used as one of the earliest steps in the diagnoses of a number of pathological conditions. Constant exposure to high concentrations of oxygen radicals, the lack of nucleus and mitochondria, inability to synthesize the new proteins and degradation of detoxifying enzymes make red blood cells (RBCs) uniquely vulnerable to oxidative stress [6]. Owing to its importance, any abnormality in the red blood cell has major consequences.

Membrane lipids and proteins are considered crucial in the maintenance of the RBC's shape. Even minimal changes in the surface area may lead to morphological and functional abnormalities which can cause pathological consequences [7]. Considerable evidence indicates that the generation of free radicals increases the red cell damage/ hemolysis by causing the membrane lipid peroxidation and protein glycation [8]. It has been proposed that stress induced protein glycation and its product accumulation participate in the cellular and tissue damage [9, 10]. A combination of oxidative stress and protein glycation underline most cases of diabetes, renal failure and coronary heart diseases like atherosclerosis [11]. Hence, any compound / medicinal plant which can inhibit the free radical generation and its induced lipid peroxidation and protein glycation may open the new therapeutic target for the treatment of red cell related pathological consequences.

Isoflavones, mainly derived from soybean are a group of biologically active substances with a chemical structure similar to that of estrogen [12, 13] . Evidence suggests that the isoflavones derived from soybean has a potential antioxidant property [14]. Since the compound is able to scavenge free radicals, they are believed to have potential in the prevention of atherosclerosis, diabetes and their complications [15]. Recently, some studies reported that soy isoflavones improve diabetic

complications through their anti-oxidative, mild estrogenic activity [16]. In contrast data from some interventional studies have failed to show an antioxidant effect from soy isoflavones supplementation [17–19]. Research supports the concept that soy intake aids against oxidative stress as indicated by the measurements of conjugated dienes in LDL fraction [20]. Still it is controversial whether isoflavones have antioxidant property or not and the problem has not been adequately addressed. In lights of the above in literature, we have made an attempt to evaluate the antioxidant property of isoflavones extracted from soybean hypocotyls by assessing its protective effect on red blood cell damage as well as plasma protein degradation induced by oxidative stress.

II. Materials and Methods

Sample preparation: Blood samples were collected into tubes containing disodium salt of ethylenediaminetetraacetate (EDTA-disodium salt) from healthy normal human volunteers after overnight fasting of 10-12hrs. The sample was centrifuged (6000rpm, 10mins at 4^0C); the clear plasma and buffy coat layers were discarded. Then, the red cell suspension was washed three times with cold saline (0.15 M sodium chloride) and the cells were suspended to 10 % haematocrit in phosphate buffered saline (PBS containing 0.016mol/L Na_2HPO_4, 0.001 mol/L NaH_2PO_4 and 0.14 mol/L NaCI, pH 7.4). This study was approved by institute human ethic committee and written consent form was obtained by the participants.

Extraction of isoflavones from soybean hypocotyls (*Glycine max*): Isoflavones were extracted from soybean hypocotyls according to the method described by Yoon-Bok Lee et al [21] . The soybean hypocotyls were mixed with 10 volumes of 80% aqueous methanol and stirred for 4hr at room temperature. The methanol extract was concentrated in a rotary evaporator at 50^0C. The final step of the preparation involved freeze – drying the concentrated methanol extract. The Isoflavone extract was stored at -40^0C for further analyses.

Assessment of free radical scavenging capacity of Soy Isoflavones extract:

2, 2'- dipicryl 2'- phenylhydrazine (DPPH) assay: The free radical scavenging capacity of soy isoflavones was assessed by 2, 2'- dipicryl 2'- phenylhydrazine (DPPH) assay [22]. A standard graph was plotted using Gallic acid as a standard in the range of 0 - 100µM/L concentration. It was found that the free radical scavenging capacity of soy isoflavones was 6.23 ± 1.04 µM/L equivalents of Gallic acid.

***In vitro* treatment with high glucose concentration:** All the glass wares and reagents used for this experiment were autoclaved prior to use to prevent the hemolysis. Washed red blood cells were suspended to 10% haematocrit in phosphate buffered saline (PBS containing 0.016mol/L Na_2HPO_4, 0.001 mol/L NaH_2PO_4 and 0.14 mol/L NaCI, pH 7.4). Aliquots of the cell suspension were placed in autoclaved RIA tubes, after which a freshly prepared stock solution of glucose/soy isoflavones mixture was added. Concentrations are expressed in terms of total cell suspension. Red cells treated with 5mmol/l glucose were considered as control. The tubes were incubated at 37^0C for 24hrs with mild agitation. All preparations kept for incubation contained 300 µg of penicillin/ml of cell suspension to prevent any microbial growth. Glucose treated red cells were washed twice with PBS (pH 7.4) before biochemical as well as hematological analysis. All analyses were repeated six times.

***In vitro* treatment with soy isoflavones extract:** In order to examine the antioxidant property of soy isoflavone extract, red cells were pre-treated with isoflavones extract in the concentration of 60µg/ml and 120µg/ml for 1 hr at 37^0C with mild agitation. Then, the pre-treated red cells were incubated with glucose of either at 5 or 50 mmol/L at 37^0C for 24hrs.

Measurement of RBC-membrane lipid peroxidation (RBC-MDA) and reduced glutathione (RBC-GSH): Malondialdehyde was measured using the established thiobarbituric acid (TBA) method [23]. This assay is based on the formation of red adduct in acidic medium between thiobarbituric acid and malondialdehyde, a colorless product of lipid peroxidation, measured at 532nm. The MDA values were calculated using the extinction coefficient of MDA-TBA complex (1.56×10^5 l X mol $^{-1}$ X cm $^{-1}$ at 532nm). The erythrocyte reduced glutathione content was determined by the method of Beutler et al [24]. The hemoglobin content of the cell suspension was estimated using Drabkin's solution following manufacturer's instructions. The reduced glutathione values were expressed as mg/g Hb.

Estimation of Glycated hemoglobin (HbA1c) and glucose: The glucose levels were estimated using glucose oxidase method and glycated hemoglobin HbA1c was measured immunoturbidometrically using Olympus AU400 fully automated Clinical Chemistry Analyzer (Bayer's Diagnostics, USA).

Measurement of red cell count and RBC indices: The red cell count and RBC indices like hemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and red cell distribution width (RDW-SD) were estimated using reagent kits adapted for the fully automated blood cell count analyzer (XS-1000*i* ^{TM}, SYSMEX, Japan).

Microscopic analysis of red cell morphology: Microscopic characteristics of red cells of different groups were observed using CKX 31 Standard Cell Culture Inverted Microscope System (Olympus, Japan). The magnitude of 400X was used for all observations.

***In vitro* treatment of plasma with AAPH:** The plasma was diluted with normal saline (1:1, V/V) and were treated with AAPH for 2 hours at 37^0C. AAPH, a compound which produce free radicals when dissolved in the medium. In order to examine the antioxidant property of soy isoflavone extract, the plasma were pre-treated with isoflavones extract in the concentration of 30µg/ml, 60µg/ml and 120µg/ml for 1 hr at 37^0C.

Measurement of plasma parameters: After incubation period, the plasma protein carbonyl content was estimated by the method described previously. The plasma malondialdehyde was measured by the method of Yogi et al. The plasma proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Bio – Rad, China). The total plasma protein and albumin levels were measured using reagent kits adapted for fully Automated Clinical Chemistry Analyzer (Olymbus AU400, Japan) according to the manufacturer's instruction.

Statistical analysis: Results were expressed as mean ± SD unless otherwise stated. Data were analyzed statistically using *one way ANOVA analysis* followed by *Tukey post-Hoc method*. The Statistical Package of Social Service (SPSS version 16 software) was used for all analysis. A P value less than 0.05 was considered as significant.

III. Results

The effect of soy isoflavones on hemoglobin glycation is shown in Fig 1. High concentration of glucose caused an increase in HbA1c levels. The increase in HbA1c was inhibited by supplementation with isoflavones extract. Additionally, the inhibitory effect of isoflavones on protein glycation was greater with increasing concentrations of isoflavones in cell suspension.
In Fig 2, the glucose level was significantly higher in the groups treated with 50mM glucose than 5mM glucose. But the glucose concentration in the supernatants of 50mM glucose treatments was found to be significantly reduced when pre-incubated with isoflavones. The inhibitory effect of isoflavones on the supernatant glucose concentration was proportional to the concentration of isoflavones. However, the above mentioned observations were not found with erythrocytes incubated with 5mM glucose.

Fig 3 shows that the effects of isoflavones on lipid peroxidation (MDA) in erythrocytes in the presence of 5mM and 50mM concentrations of glucose. The MDA levels were significantly elevated in red cells treated with 50 mM glucose than the cells treated with 5mM glucose. Treatment with isoflavones reduces the MDA levels significantly and this reduction was greater with increasing the concentration of isoflavones. Furthermore, the isoflavones did not have any significant effect on reduced glutathione levels in all the groups (fig 4)

Table1 showed that, the red cell count and hemoglobin concentration were significantly decreased in RBC treated with 50mM glucose concentration when compared to the 5mM glucose concentration. The effect was inhibited when the RBCs were pre-incubated with soy isoflavones at 60µg and 120µg concentrations. The soy isoflavones did not have any significant effect on other RBC indices like MCH, MCV, MCHC and RDW-SD.

Fig 5 shows the effect of soy isoflavones extract on red cell membrane damage after generating the oxidative stress using 50mM glucose. Increase in oxygen radicals leads to red cell membrane damage resulting in hemolysis. In this present study, we found that the cell membrane rapture and damage in the groups treated with pathological concentration of glucose (50mM) was higher when compared to the groups treated with physiological concentration (5mM). Pre-incubation with 120µg/ml soy isoflavones might have reduced plasmas membrane damage from free radicals. However, no significant effect was found at the concentration of 60µg/ml of isoflavones.
The plasma protein carbonyl content and malondialdehyde were significantly increased and total protein and albumin levels were significantly reduced when plasma was treated with AAPH. Supplementation of soy isoflavones extract reduced the plasma malondialdehyde and protein carbonyl contents (Table 2). Electrophoresis of plasma protein revealed that AAPH treatment significantly caused protein degradation and this effect was blocked when plasma was pre-incubated with soy isoflavones (Fig 6).

IV. Discussion

Exposure to high concentrations of oxygen radicals, the lack of nucleus and mitochondria, inability to synthesize new protein and degradation of detoxifying enzymes makes red blood cells uniquely vulnerable to oxidative stress. In the laboratory, red blood cells are a reliable model for the study of oxidative stress. They are one of the first cells to be affected by adverse conditions and any alteration in their structure causes pathological consequences [1, 5]. In this present study, we therefore selected RBCs as an oxidative stress model to study the protective effects of soy isoflavones against oxidative stress. The free radicals were generated by exposing the RBCs with higher concentration of glucose (50mM). Higher glucose concentration can result in increased oxidative stress from excessive oxygen radical production from the auto-oxidation of glucose, glycosylated protein, or stimulation of cytochrome P450 - like activity by the excessive NADPH produced by the glucose metabolism [25].

Higher concentration of glucose can promote the glycation of several proteins of RBCs including hemoglobin by non-enzymatic mechanisms [26]. In our study, we found that the hemoglobin glycation as indicated by the levels of HbA1c were significantly elevated at pathological concentrations of glucose (50 mM) when compared with cells exposed to physiological concentrations (5mM). The increased glycation of proteins themselves can act as a source of free radicals [26] The increased production of free radicals change lipid/protein ratio of membranes by affecting polyunsaturated fatty acids. A lipid peroxidation causes functional irregularities of several cellular organelles [27]. Treatment with soy isoflavones significantly reduced the hemoglobin glycation (HbA1c) in the group treated with high concentration of glucose. This inhibitory effect of isoflavones on protein glycation may help in the reduction of glycation induced free radical generation. Furthermore, we also observed that soy isoflavones have a potential ability to scavenge the free radicals which was indicated by the DPPH assay. This free radical scavenging capacity of isoflavones might have contributed to its ability in reducing the supernatant glucose concentration as well as in inhibiting further protein glycation. Furthermore, these effects may bring down the free radical generation which follows a vicious cycle.

Lipid peroxidation is another free radical-related process, which is potentially harmful because of its uncontrolled, self-enhancing nature. This causes disruption of bio-membranes, lipids and other cell components [27]. Malondialdehyde, an end product of lipid peroxidation is increased when the cells are exposed to oxidative stress. We found significant increase in the level of MDA in red cells with pathological concentration of glucose when compared to the red cells with physiological concentration of glucose. Research supports the concept that malondialdehyde (MDA) can promote the process of protein glycation. But the mechanism is not clearly addressed. It is presumed that the aldehyde groups of malondialdehyde act as an anchor between sugar and protein moieties, thereby enhancing

the formation of glycated proteins [28]. Treatment of red cells exposed to pathological concentration of glucose with isoflavones significantly reduced the MDA levels. This might have helped in subsequent inhibition of protein glycation and its induced free radical generation.

The microscopic analysis of RBCs shows that there was a significant morphological abnormality in the membrane of RBCs exposed to high glucose (50mM) concentration than the cells exposed to normal concentration of 5mM glucose. These morphological changes could be attributed to the cumulative effects of increased membrane lipid peroxidation and protein glycation promoted by oxidative stress. However, the stress induced damage of RBCs was not observed in the group pre-treated with soy isoflavones at the concentration of 120 µg/ml, but not at 60 µg/ml. Treatment with isoflavones significantly decreased both the protein glycation and lipid peroxidation and this might have protected the RBCs against oxidative damage. Furthermore, we estimated the red cell count after the treatment period and found a significant reduction in the red cells in the group treated with pathological concentrations of glucose. The enhanced membrane lipid peroxidation and glycation of protein might have resulted in membrane damage and subsequent cell death. Treatment with isoflavones markedly increased the red cell count. The increase in the red cell count might have improved the glucose utilization and resulted in the reduction in the supernatant glucose after incubation.

Furthermore, the antioxidant property of soy isoflavones extract was further evaluated using plasma as a invitro model of oxidative stress. The plasma was incubated with free radical generating compound known as AAPH. When the plasma was treated with AAPH, the plasma malondialdehyde and protein carbonyl contents were increased and total protein and albumin levels were reduced. Treatment with soy isoflavones extracted significantly reduced the plasma malondialdehyde and protein carbonyl contents. In lights of the above results in RBC, the results of plasma also supporting the evident that soy isoflavones have the potent antioxidant property which might help in protecting the red cells and plasma protein degradation from oxidative stress.

Taken together, the results of the present study concluded that, the soy isoflavones extract protect red blood cells and plasma protein degradation from oxidative stress. This protective effect of isoflavones extract on red cells may be due to the inhibition of protein glycation and membrane lipid peroxidation by its antioxidant properties.

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AUTHORS

First Author – Author name, qualifications, associated institute (if any) and email address.
Second Author – Author name, qualifications, associated institute (if any) and email address.
Third Author – Author name, qualifications, associated institute (if any) and email address.

Correspondence Author – Author name, email address, alternate email address (if any), contact number.

Figures:

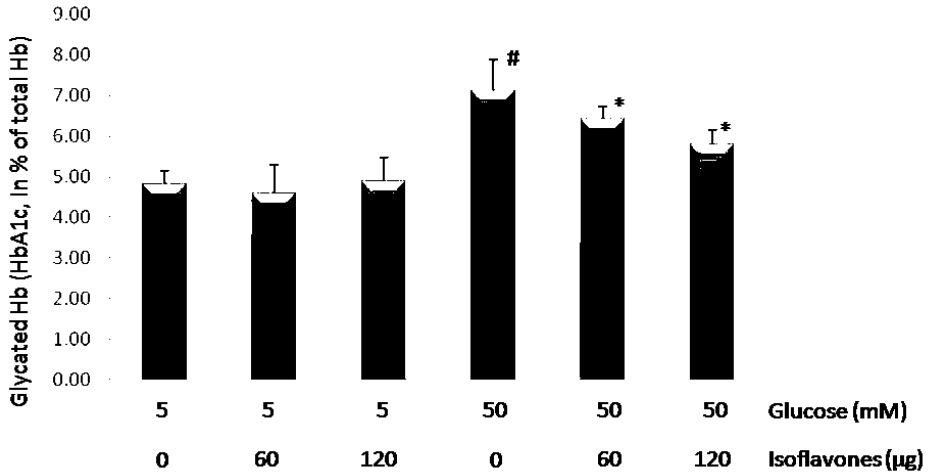

Fig 1: Effect of soy isoflavones extract on hemoglobin glycation (HbA1c). Values are Mean ± SD (n=6). # P < 0.05 when compared to incubations with 5mM glucose concentration. * P < 0.05 when compared to the incubation with 50mM glucose concentration in the absence of isoflavones. (One way analysis of variance with Tukey post hoc test, SPSS 19.0)

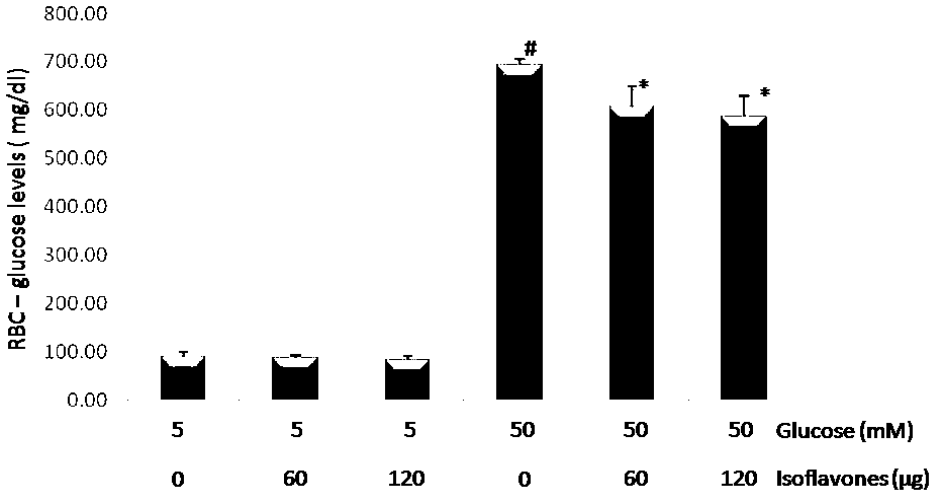

Fig 2: Effect of soy isoflavones extract on the glucose concentration in the medium after incubation. Values are Mean ± SD (n=6).# P < 0.05 when compared to incubations with 5mM glucose concentration. * P < 0.05 when compared to the incubation with 50mM glucose concentration in the absence of isoflavones. (One way analysis of variance with Tukey post hoc test, SPSS 19.0)

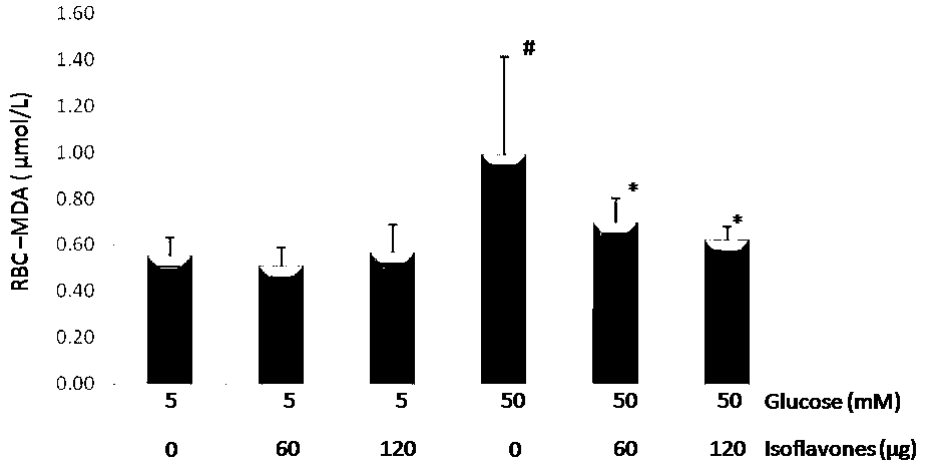

Fig 3: Effect of soy isoflavones extract on lipid peroxidation (RBC - MDA) in RBC treated with different concentrations of glucose. Values are Mean ± SD (n=6). # P < 0.05 when compared to incubations with 5mM glucose concentration. * P < 0.05 when compared to the incubation with 50mM glucose concentration in the absence of isoflavones. (One way analysis of variance with Tukey post hoc test, SPSS 19.0)

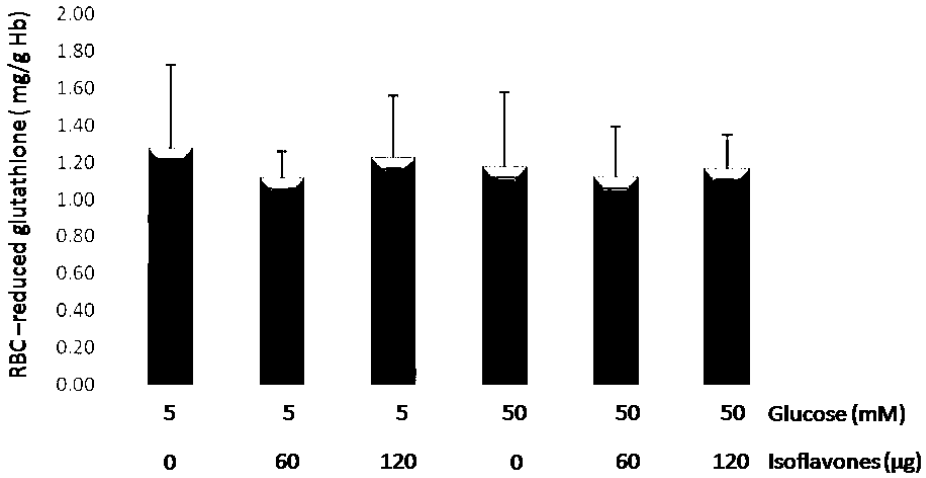

Fig 4: Effect of soy isoflavones extract on RBC reduced glutathione (RBC-GSH) concentration. Values are Mean ± SD (n=6). (One way analysis of variance with Tukey post hoc test, SPSS 19.0)

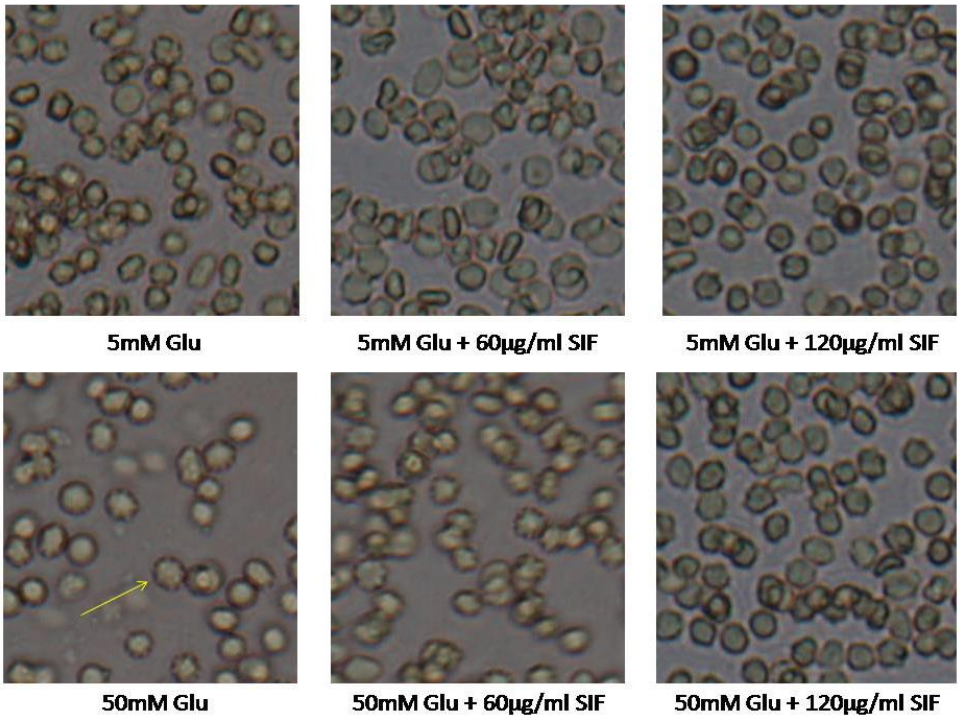

Fig 5: Effect of soy isoflavones extract on RBC morphology in 5mM and 50mM glucose treated red cells. The red cells images were observed using CKX 31 Standard Cell Culture Inverted Microscope System (Olympus, Japan). The magnitude of 400X was used for all observations.

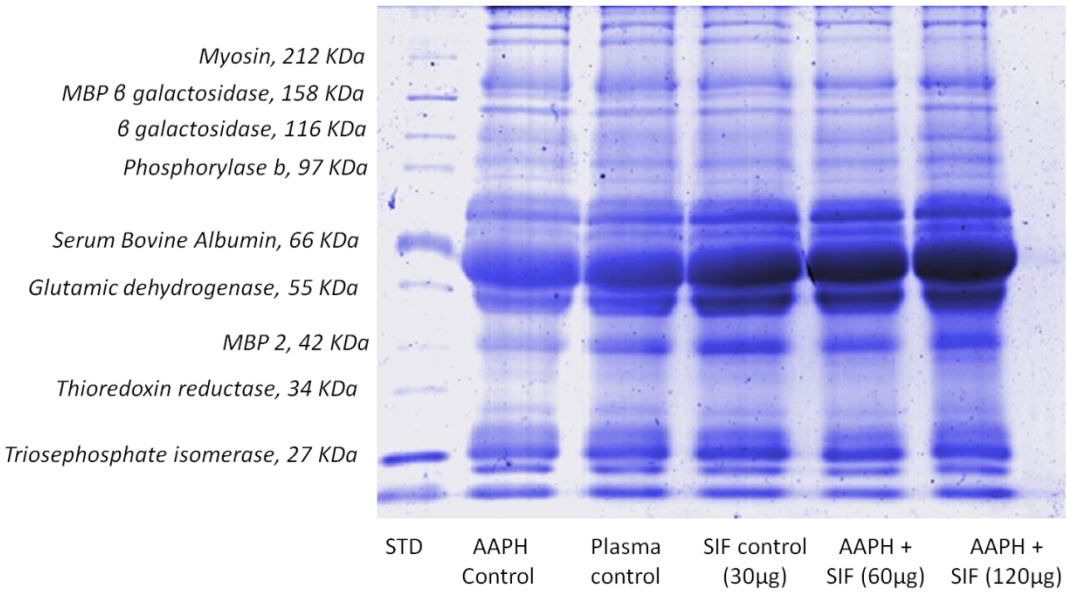

Fig 6: SDS PAGE separation of plasma protein. Ten percentage separating gel was used for separation. AAPH treatment caused reduced total proteins and albumin levels and this was inhibited when plasma was pre-treated with soy isoflavones extract. Image was captured in Image Densitometer GS 710 (Bio Rad, China) and analysis were done using Quantity One software.

Table 1: Effect of soy isoflavones extract on red cell count and RBC indices

Parameters	5mM Glu	5mM Glu + 60µg SIF	5mM Glu + 120µg SIF	50mM Glu	50mM Glu + 60µg SIF	50mM Glu + 120µg SIF
RBC count (millions/m^3)	0.84±0.04	0.84±0.03	0.84±0.02	0.76±0.02 *	083±0.02 #	0.84±0.01 #
Hb (g%)	2.33±0.05	2.38±0.04	2.37±0.05	2.13±0.05 *	2.37±0.05 #	2.37±0.05 #
MCV (fl/cell)	92.73±0.12	91.97±1.58	93.13±1.45	93.05±0.58	92.87±0.94	92.72±0.74
MCH (pg/cell)	28.18±0.79	29.05±0.55	28.62±0.65	28.58±0.38	28.75±0.66	28.88±0.91
MCHC (%)	30.52±0.80	31.22±0.78	31.02±1.14	30.63±0.40	31.07±0.88	30.65±1.2
RDW – SD	39.7±0.70	39.2±1.05	39.3±0.98	39.5±0.70	39.8±0.61	39.7±0.34

Glu – Glucose, SIF – Soy isoflavones, Hb – Hemoglobin, MCV – Mean corpuscular volume, MCH – Mean corpuscular hemoglobin, MCHC – Mean corpuscular hemoglobin concentration, RDW-SD – Red cell distribution width (standard deviation)
Values are Mean ± SD (n=6). * $P < 0.05$ when compared to incubations with 5mM glucose concentration.# $P < 0.05$ when compared to the incubation with 50mM glucose concentration in the absence of isoflavones. (One way analysis of variance with Tukey post hoc test, SPSS 19.0).

Table 2: Effect of soy isoflavones extract on plasma malondialdehyde and protein carbonyl contents

Parameters	MDA	PCO	TP	Alb
Plasma Control	9.38 ± 0.21	7.88 ± 0.48	9.04 ± 0.74	5.09 ± 0.31
AAPH Control	12.61 ± 0.75 [a]	8.68 ± 0.42 [a]	7.68 ± 0.45 [a]	3.63 ± 0.52 [a]
SIF (30µg) control	7.91 ± 0.38	7.68 ± 0.46	8.73 ± 0.49	5.15 ± 0.18
SIF (60µg) control	8.19 ± 0.85	7.43 ± 0.89	7.92 ± 1.12	5.11 ± 0.2
SIF (120µg) control	8.23 ± 0.55	7.05 ± 0.64	8.58 ± 0.58	4.77 ± 0.73
AAPH + SIF (30µg)	12.88 ± 0.55	8.03 ± 0.73	7.55 ± 0.63	3.88 ± 0.41
AAPH + SIF (60µg)	11.08 ± 0.54	7.58 ± 0.78	8.26 ± 0.22	4.44 ± 0.29
AAPH + SIF (120µg)	8.99 ± 0.5 [b]	6.83 ± 0.7 [b]	8.55 ± 1.26 [b]	4.91 ± 0.17 [b]

MDA – malondialdehyde, SIF – Soy isoflavones, PCO-protein carbonyl content, TP – total protein, Alb-albumin
Values are Mean ± SD (n=6). [a,b] $P < 0.05$ statistically significant. a – in comparison with control. b – in comparison with AAPH control. (One way analysis of variance with Tukey post hoc test, SPSS 19.0)