Pomegranate Juice Improves Iron Status and Ameliorates Iron Deficiency Induced Cellular Changes in *Saccharomyces cerevisiae*

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Abstract: *Background*: Iron Deficiency Anemia (IDA) is most prevalent form of anemia affecting around 2 billion people world-wide. Ayurveda, an Indian system of medicine, describes pomegranate (*Punica granatum*) fruits as a *Rasayana* and a dietary supplement for managing a condition called *Pandu*, which is akin to IDA. *Rasayanas* are methods to maintain homeostasis by improving digestion, metabolism and absorption of nutrients and elimination of waste. Yeast (*Saccharomyces cerevisiae*) has been a well-accepted model organism to study iron metabolism.

Materials & Methods: In the current study we developed 'anemic yeast' by culturing yeast cells in iron-free medium with bathophenanthroline disulfonate (BPS). The effect of pomegranate juice (PJ) on reversing the 'IDA like' condition in yeast was studied.

Results: Culturing iron deficient (ID) cells in the presence of 10% PJ supplemented medium (IDP), improved iron status by at least 7 fold ($p<0.0001$) and reversed mitochondrial degeneration induced by iron deficiency. Percentage of healthy reticulate mitochondria in IDP cells was >30% higher ($p<0.0001$) than that in the ID cells grown in iron deficient medium (IDD) and at least 14% more than that in ID cells grown in 10% PJ-equivalent iron substituted media. Interestingly, PJ substitution improved the functional ferrous (Fe^{2+}) form as well as the bio-assimilated heme form of iron, but not the ferric (Fe^{3+}) storage form in ID cells.

Conclusion: Yeast model can be useful as a quick screen to identify potential nutritional supplements. Pomegranate's potential role as a nutritional supplement in IDA management and as a hematinic is worthy of further research.

Keywords: Iron deficiency anemia, Ayurveda, Pandu, Pomegranate, Rasayana, *S. cerevisiae.*

INTRODUCTION

Iron is an essential micronutrient involved in a variety of biological processes of cells and plays a role in oxygen transfer [1]. Iron deficiency occurs when there is iron loss, insufficient intake or the bio-available iron is inadequate to meet the body iron demands, resulting in low haem concentrations [2]. Iron deficiency anemia (IDA) is a common disorder affecting about 2 billion people mostly women and children [3]. Iron is a transition metal and exists in the reduced ferrous (Fe^{2+}) and oxidized ferric (Fe^{3+}) forms. Reduction reactions play a crucial role in iron metabolism, because only reduced iron can be transported across the membrane, load and release iron from ferritin and contribute to heme synthesis [1, 4].

Programs to increase the bioavailability of iron through the use of iron supplementation as pills, iron bio-fortification, use of iron bio-availability enhancers like ascorbates, citrates and folic acid have not been very successful in creating significant impact due to possible side effects including nausea and constipation [5, 6]. Currently researchers are attempting to demonstrate the use of greens, herbs, fruits and vegetables as food and beverage to support IDA management [7-10]. Interventions guided by traditional knowledge on dietary supplements to manage IDA may provide ecosystem specific natural materials that are cost-effective and culturally acceptable.

Ayurveda, a traditional medical system of India has indicated several herbs and fruits that can be used in the management of '*Pandu*', a disease condition correlated to IDA [11]. Ayurvedic classical texts describe pomegranate as a Rasayana with 'wholesome' (*Pathya*) properties, abilities to induce tissue generation, development (*Dhatuvrddhikara*), strength promotion (*Balya*) and is also prescribed for use in cardiac and anemic conditions [12]. Rasayanas are rejuvenating methods that improve digestion, metabolism and tissue perfusion of nutrients [13, 14].

Pomegranate (*Punica granatum* L.), a member of the Punicaceae family is a commercially cultivated fruit

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in many countries and available pan-globally throughout the year. It is rich in polyphenols and organic acids [15]. Apart from glucose, fructose and sucrose, the juice contains ellagic acid, gallic acid, anthocynidins, flavanols, ellagitannins, straight chain fatty acids, citric acid and malic acid [15]. Pomegranate juice is also reported to be rich in essential mineral nutrients like iron, phosphorus, copper, sodium, magnesium, potassium, calcium, zinc and manganese [16]. Pomegranate has been reported to have various bioactivities like anti-inflammatory, anti-oxidant and has also shown to be effective in cardiovascular disease, diabetes, male infertility, cancer, Alzheimer's disease, arthritis and obesity [17, 18]. Recently the healthy life-span enhancing property of pomegranate juice was demonstrated in drosophila model [19].

Several *in vitro*, *in vivo* and *ex vivo* models have been developed to study iron metabolism under laboratory conditions [20]. The budding yeast *Saccharomyces cerevisiae* has provided significant insight into iron metabolism in eukaryotes. Iron metabolism pathways are conserved from *S. cerevisiae* to humans [21]. Effect of natural products like desferroxamine [22], curcumin [23] and sampangine [24] on cellular iron metabolism has been elucidated using *S. cerevisiae* as model. Iron chelator, BPS (Bathophenanthroline disulfonic acid disodium salt hydrate) has been used in several studies to induce iron deficiency leading to the formation of 'iron deficient' yeast cells' [25]. In *S. cerevisiae* iron deficiency was found to alter metabolic pathways, limit growth and reduced energy production in mitochondria [26].

The present study was designed to understand the role of pomegranate juice in ameliorating iron deficiency induced cellular changes in *S. cerevisiae* model. We hypothesized that pomegranate juice can improve the iron status and cellular physiology in experimentally generated anemic yeast cells.

MATERIALS AND METHODS

Saccharomyces cerevisiae Strain and Culture Conditions

Wild type *S. cerevisiae* strain (BY4742; Genotype: MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) obtained as a kind gift from Prof. Matt Kaeberlein's lab, University of Washington in Seattle, USA was used for the study. Cells were routinely grown in the SD medium that composed of yeast nitrogen base (YNB, 1.7 g/L; Himedia, Mumbai) and complete supplement mixture (CSM, 0.79 g/L; Himedia, Mumbai). All cells were grown by incubating at 28 ºC in a shaking incubator (180 RPM; Remi, Mumbai) for 12 to 14 hrs.

Iron deficiency was induced by growing the *S. cerevisiae* cells in synthetic defined (SD) medium without iron (calling it D medium) and also by incorporating 100µM BPS (Sigma-Aldrich, MO, USA) [23, 25].

Generation of Mitochondrial sm GFP *S. cerevisiae* Cells

Yeast expression vector (p426 GPD) with GFP linked to mitochondrial localization sequence, isovaleryl-CoA-dehydrogenase (IVD) was obtained by sub-cloning the encoding cDNA (IVD-*GFP) from a plant expression vector [27]. Full length IVD-GFP was cut by using Xba I and EcoRI enzymes (New England Biolabs, Massachusetts) and was cloned. The constructs were transformed to BY4742 yeast strain and the clones were selected for growth on a minimal media lacking uracil. Soluble modified GFP (smGFP) positive cells were used for mitochondrial morphology assessment experiments.

Preparation and Standardization of Pomegranate Juice (PJ)

PJ was prepared by hand crushing the arils of fresh fruits through sterile muslin cloth [19]. The juice was passed through a filter (0.2 µm; Millex, Millipore, Germany) and stored as aliquots of different volume (5, 15 and 50 ml) in screw cap tubes at -80 ^{o}C until use. PJ was standardized by estimation of total phenolics, total acids and iron content (see supplementary files). The PJ once thawed was not re-frozen.

Experimental Outlay

The dose response with incorporation of pomegranate juice (5% to 50%) in medium on the growth profile of cells in SD media was studied. A concentration of 10% PJ was selected for further experiments based on the growth curve, generation time and viable cell count in complete SD medium, iron-free medium and uracil-free medium.

A loop full of yeast cells from SD agar plate was inoculated in 100 ml each of SD medium containing iron (N) and iron deficient (D) medium (ie., SD medium without iron and containing 100µM BPS) in 250 ml conical flasks until the iron normal (IN) and iron

deficient (ID) yeast cells reached mid-log phase (A_{600} of 0.35). In order to study the iron deficiency reversal potential of pomegranate, the ID cells were further cultured in different media as mentioned below:

(i) D medium: iron deficient SD medium to obtain IDD cells

(ii) P medium: D medium supplemented with 10% PJ (90µg/100ml Fe content) to obtain IDP cells

(iii) F medium: D medium with 90µg/100ml Fe (iron content equivalent of 10% PJ) as $FeCl_3$ to obtain IDF cells

(iv) N medium: SD medium (20µg/100ml Fe content) to obtain IDN cells

Dry Biomass, Cell Viability and Size

About 2 ml of broth culture containing $1x10^6$cells/ml was aliquoted into pre-weighed microcentrifuge tube, pelleted at 6000 rpm for 10 minutes and washed with sterile distilled water. After removing traces of the liquid, the pellet was dried at 60°C until constant weight. The difference in the pre and post weight of the tube was taken as the dry weight (mg) per 10^6 cells.

For cell viability studies, each culture was aseptically diluted to obtain 10^{-2}, 10^{-4} and 10^{-6} dilutions. About 5µl of each dilution was spotted individually on SD agar plates in triplicates. The plates were incubated at 28ºC for 48 h. Distinct individual colonies on each spot were counted and CFU/ml was calculated accounting for the dilution factor. Generation time / doubling time was calculated using online software tool 'Doubling Time' (http://www.doubling-time.com/compute.php) and was expressed in minutes.

For determining cell size, 10µl of the *S. cerevisiae* broth culture (14 h) was placed on a clean glass slide and focused under 100x of Olympus microscope (BX41, Tokyo) and image captured with Olympus digital camera (DP72, Tokyo) fitted to the microscope. The cell size was determined as the average diameter of 50 individual cells using calibrated Image Pro Express 6.0 software and represented as µm ± standard deviation.

Total RNA, Protein and Lipid

Total RNA and protein were extracted using TRIreagent (Sigma, MO) following manufacturer's instruction. The quantity of RNA and protein was estimated using nano-quantity spectrophotometer (Thermo scientific, DE) at 260 and 280nm respectively.

Lipid extraction was performed as per the method described by Folch *et al*., [28] with some modification. Freshly cultured cells were vigorously vortexed with 20 volumes of chloroform:methanol (2:1) (1 g in 20 ml of solvent mixture). The homogenate was filtered using Whatman filter paper no.1 to recover the liquid phase. The liquid collected was washed with 0.2 volume 0.9% NaCl solution. After vortexing, the mixture was centrifuged at 2000 rpm for 4 minutes at room temperature to separate the two phases. The lower chloroform phase containing lipids was collected in a pre-weighed (A) micro-centrifuge tube. Chloroform was evaporated by keeping the tubes open in an incubator set at 65 °C, until a constant dry weight (B) was obtained. The difference between B and A was considered as the quantity of total lipids and expressed as mg lipids per g dry weight of yeast cells.

Iron and Heme Content

Total Iron and Fe^{2+} content in the cells was estimated using colorimetric assay kit (Biovision, CA) following manufacturer's instruction. Quantity of iron in the sample was calculated using a calibration curve with known concentrations of Fe^{2+} standard provided with the kit. The difference in the Fe^{2+} content before and after reduction of the sample was considered as the quantity of Fe^{3+}. All experiments were performed in triplicates with three different sets of samples.

Heme content was estimated using colorimetric Hemin assay kit (Sigma-Aldrich, MO) following manufacturer's instruction. Heme obtained from the cells was diluted 100 times before estimation in microplate spectrophotometer (Biorad, USA). Quantity of heme in the sample was calculated from a calibration curve prepared with known concentrations of hemin provided with the kit. The experiment was performed in triplicates with two different sets of samples.

ATP Content

Total ATP was extracted and estimated using Enliten bioluminescence ATP detection kit (Promega, WI) following manufacturers instruction. The sample was diluted 50 times using 1M tris-acetate buffer (pH 7.75), before estimation. About 10 µl of sample was mixed with 100 µl rL/L (rLuciferase/Luciferin) reagent in a white 96 well microtitre plate (Thermo Fischer Scientific, MA, USA). Relative Light Units (RLU) was

measured in a microplate luminometer (Thermo Fischer Scientific, MA, USA) with 2s delay followed by 10s measurement period. ATP concentration was estimated using a calibration curve plotted with known concentrations of ATP. The experiment was performed in triplicates.

Assessment of Mitochondrial Structure

The morphology of the mitochondria was assessed at 12 and 24 hours using a fluorescence microscope (BX41, Olympus, Tokyo) fitted with a digital camera (DP72, Olympus, Tokyo) by random image capturing of about 100 cells per each sample. Each cell was manually scored by visual observation under microscope for the presence of reticulate, fragmented or clumped mitochondria [29]. The percentage of cells with each form of mitochondria per group was tabulated. The experiment was performed in triplicates with two different sets of samples.

Statistical Analysis

Student's t test was used to compare the means of two groups. Values were considered significant if $p < 0.05$ at 95% confidence limits.

RESULTS

Generation of Iron Deficient Cells

Culturing the S. cerevisiae cells in the D medium did not alter cell viability but reduced the total iron content by 89% and heme content by 39% (Supplementary Table **1**). The percentage of cells with reticulate or healthy mitochondria was significantly ($p<0.001$) lower in the ID cells when compared to IN cells (Supplementary Figure **1**).

PJ Supplementation Improved the Viability of ID *S. cerevisiae* Cells

Culturing the ID cells in iron deficient (D) medium for 14 h reduced the number of viable cells and increased the generation time (Table **1**), while it remained unchanged when the ID cells were cultured in D medium with 10% PJ substitution or in the iron normal (N) medium. IDF cells showed >50% reduced number of viable cells and 14% increase in generation time (Table **1**). The cell size remained unchanged when cultured in different media.

PJ Ameliorates ID Induced Cellular Changes

The dry weight of IDP cells was significantly ($p<0.0001$) higher when compared to that of IDD cells (Figure **1a**). The dry weight of IDP cells was almost equivalent to that of the IDF and IDN cells. IDD, IDF and IDN cells had significantly ($p<0.05$) higher RNA content than the IDP cells (Figure **1b**). However, IDP cells had significantly ($p<0.0001$) higher protein content than all the other groups tested (Figure **1c**). PJ supplementation significantly ($p<0.0001$) increased the total lipid content of ID cells when compared to IDD cells (Figure **1d**), but was far lower ($p<0.005$) than that found in IDF and IDN cells.

PJ Supplementation Improves the Iron Status of ID *S. cerevisiae* Cells

IDP cells had 7-fold higher (0.63 ± 0.007 ng/mg dry weight) iron content than IDD cells (Figure **1e**). However, unlike in the IDN cells which contained ferrous (Fe^{2+}), ferric (Fe^{3+}) and heme forms of iron, only Fe^{2+} and heme forms could be detected in IDP cell. IDN and IDF cells had significantly ($p<0.0001$) higher total iron content, in both ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms (Figure **1e**), when compared to IDD or IDP. The heme content in the IDP cells was the highest, being significantly ($p<0.0001$) higher than that in cells from any of the other experimental groups (Figure **1f**).

PJ Improves Mitochondrial Structural Composition and Function

When the mitochondrial structure of the cells grown in the different media was analyzed, IDP cells had the

Table 1: Culture Characteristics of ID *S. cerevisiae* Cells Cultured in Different Media

Group	IDD	IDP	IDF	IDN
Viability (CFU/ml)	7.6×10^5	1.0×10^6	4.8×10^5	1.0×10^6
Generation time (Min)	101.84	97.18	110.75	97.18
Cell size (µm)	1.43±0.20	1.41±0.12	1.42±0.12	1.48±0.28

ID - Iron deficient.
IDD - ID *S. cerevisiae* cultured in ID medium.
IDP - ID *S. cerevisiae* cultured in SD medium with 10% PJ.
IDF - ID *S. cerevisiae* cultured in SD medium containing Fe equivalent of 10% PJ.
IDN - ID *S. cerevisiae* cultured in SD medium.

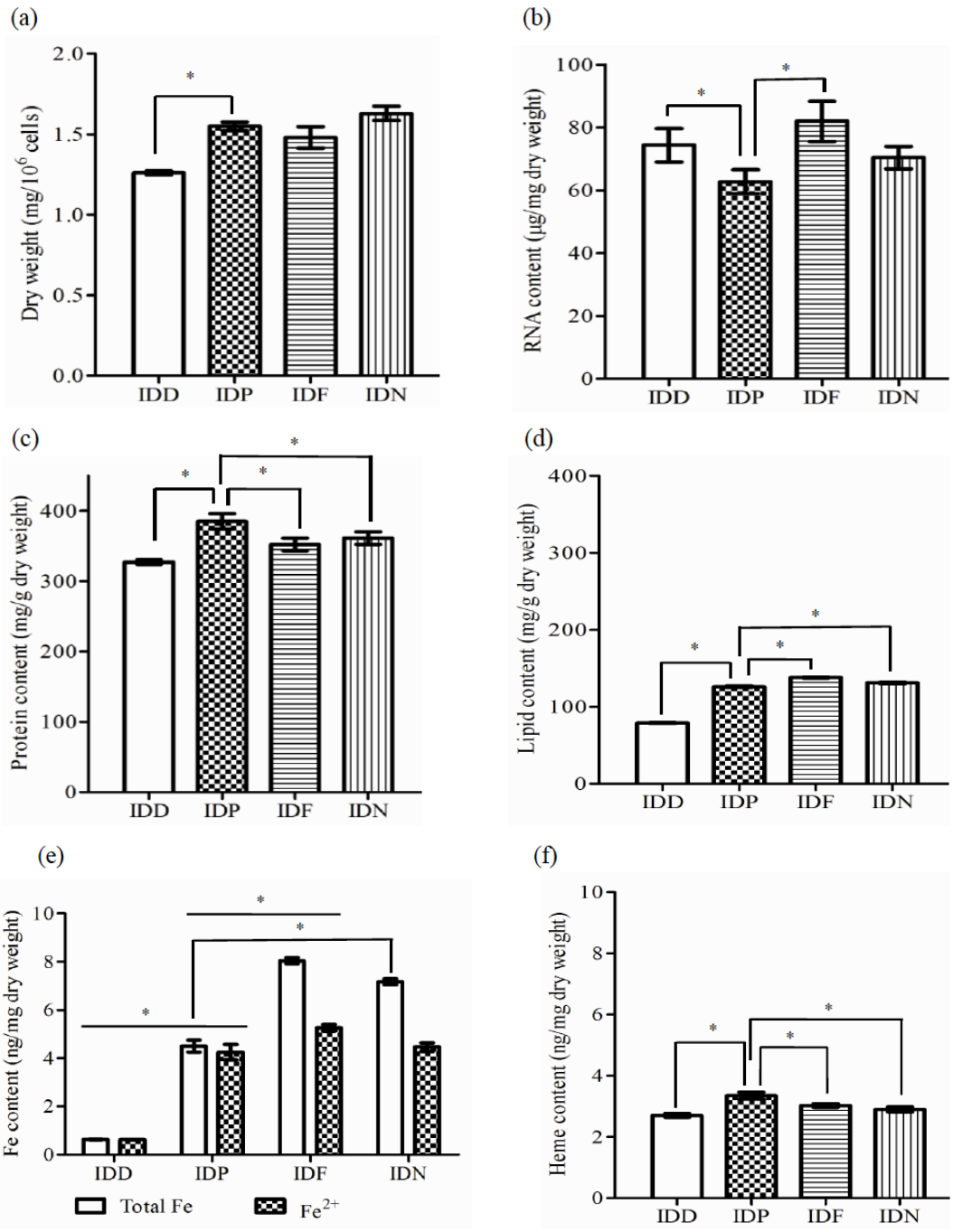

Figure 1: Effect of PJ substitution on the dry weight, RNA, protein, lipid, iron and heme content of ID *S. cerevisiae* cells.

ID - Iron deficient.

IDD - ID *S. cerevisiae* cultured in ID medium.

IDP - ID *S. cerevisiae* cultured in SD medium with 10% PJ.

IDF - ID *S. cerevisiae* cultured in SD medium containing Fe equivalent of 10% PJ.

IDN - ID *S. cerevisiae* cultured in SD medium.

*indicates statistical significance ($p<0.05$).

highest percentage of cells with healthy reticulate mitochondria at both 12 and 24 hrs (Figure **2a**). The difference was significant ($p<0.0001$) at both the time points when compared to IDD cells. While IDN cells also showed a similar trend like IDP, IDF had significantly ($p<0.005$) lower percentage of reticulate mitochondria than the IDP cells at both 12 and 24 hrs (Figure **2a**). Even though the ATP content in the IDP

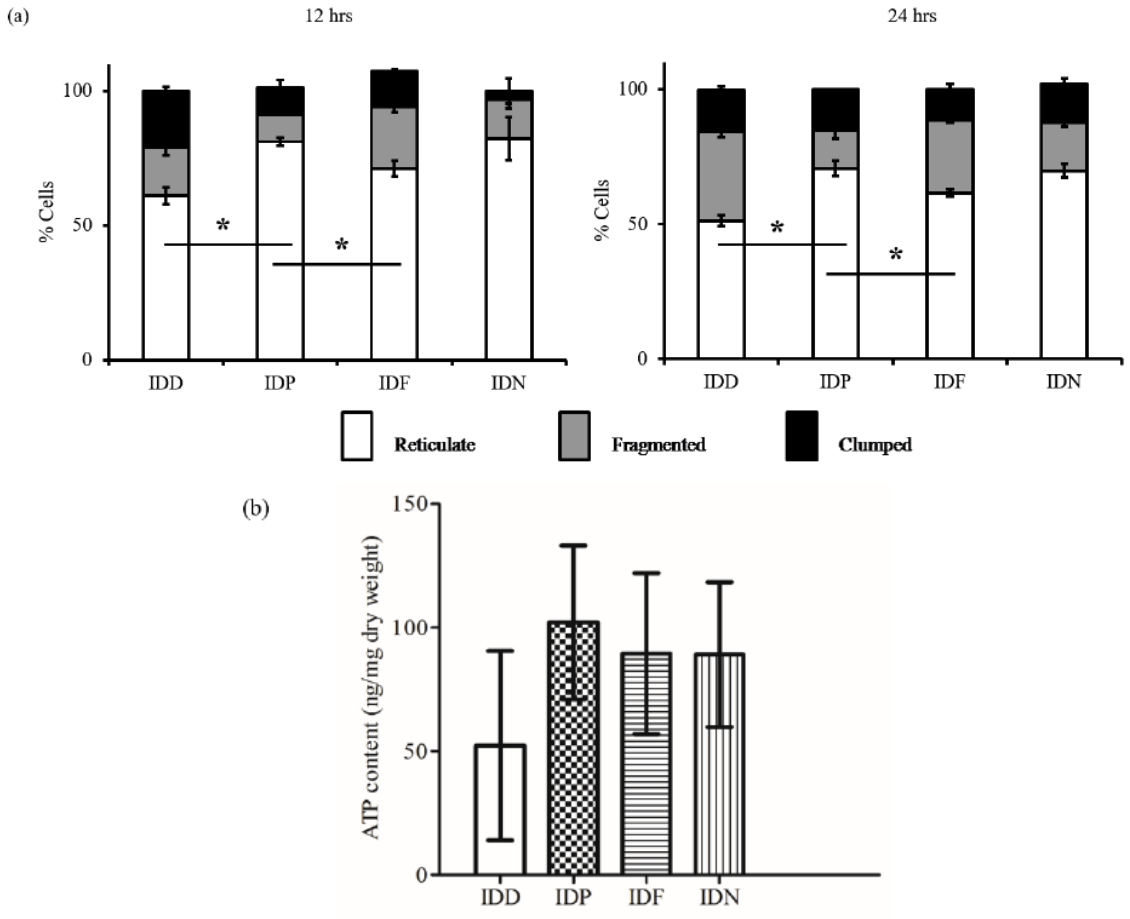

Figure 2: Effect of PJ substitution on mitochondrial structure (**a**) and function (ATP content) (**b**) of ID *S. cerevisiae* cells.

ID - Iron deficient.

IDD - ID *S. cerevisiae* cultured in ID medium.

IDP - ID *S. cerevisiae* cultured in SD medium with 10% PJ.

IDF - ID *S. cerevisiae* cultured in SD medium containing Fe equivalent of 10% PJ.

IDN - ID *S. cerevisiae* cultured in SD medium.

*indicates statistical significance ($p<0.05$).

cells was found to be higher than the cells of other experimental groups (Figure **2b**), the difference was not statistically significant.

DISCUSSION

IDA is a serious global problem despite ongoing national / international programs for decades with Fe-Fol (iron-folic acid) tablets [30]. As per Ayurveda, an Indian traditional medicine, one of the main causes of '*Pandu*' (~IDA), is mal-absorption due to '*mandagni*' (low digestive fire) [31]. Several fruits, vegetables and herbs are prescribed as dietary supplements in the management of *Pandu,* which are said to correct problems of digestion and absorption [11]; pomegranate being one such fruits [31]. In this study, the effect of PJ on reverting IDA-like condition was simulated in yeast model. With similarities in the iron metabolism pathways of humans, the single cell eukaryote, yeast has been unraveling the response of cells to availability of iron [32].

PJ induced changes in the IDD cells as observed in the current study have been summarized in Figure **3**. While iron supplementation in the medium (IDF) itself reversed the iron deficient status of ID cells, PJ improved the iron status substantially in IDP cells, particularly of the Fe^{2+} form rather than the stored Fe^{3+} form. Normally, the iron obtained is primarily used for physiological purposes in the ferrous form, excess iron is stored in vacuoles in ferric form [33]. While the N media had the required optimal iron concentration (20 µg/100ml), the P and F media contained 4.5 fold higher iron content (90 µg/100ml). Our observations indicate

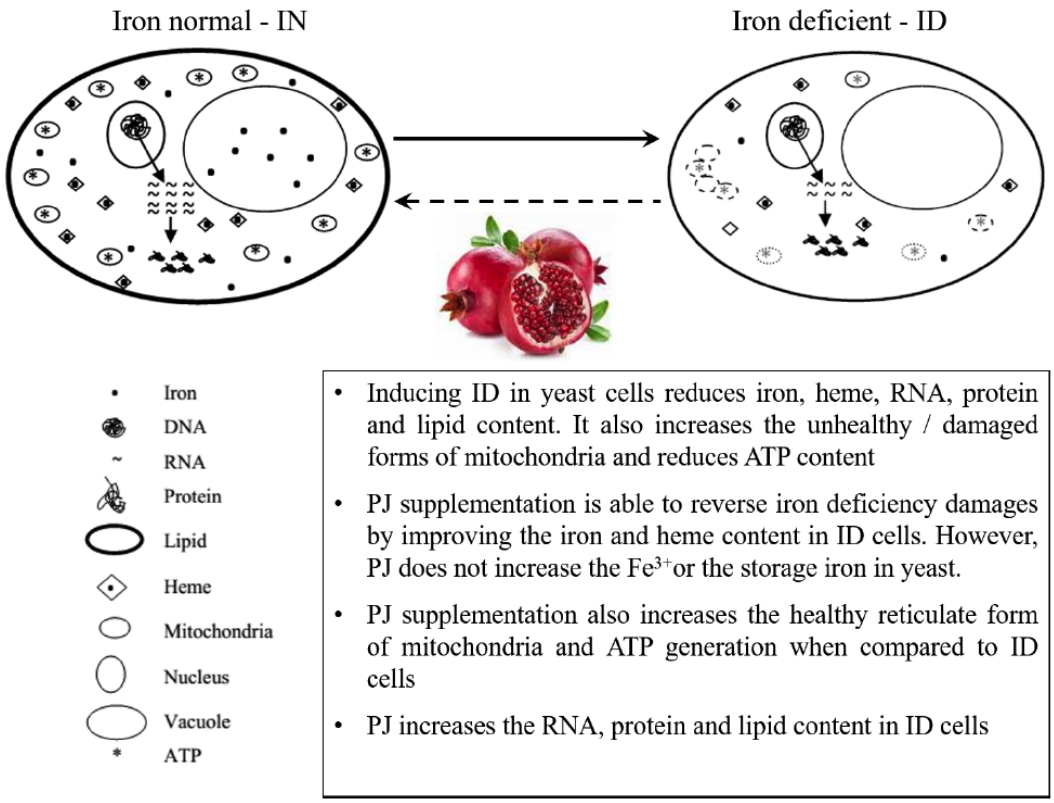

Figure 3: Diagrammatic representation of iron deficiency induced cellular changes in *S. cerevisiae*, and its amelioration by pomegranate juice (PJ).

that the storage iron (Fe^{3+}) improved only in the IDN and IDF cells and not in the IDP cells. The fact that the IDP cells showed the presence of only ferrous iron (Figure **1e**) could be due to the reducing environment in the presence of PJ [17]. Moreover, the bio-assimilated heme form of iron was significantly higher in IDP than that in other experimental groups. Increase in the heme content would have positive implications in iron uptake [24]. PJ appears to enhance heme production, as observed by the higher heme levels in IDP cells when compared to that in IDD, IDF or IDN cells. Even though there was significant increase in both functional and storage iron in the IDF cell, viability of these cells decreased drastically by 50%. Holmes-Hampton *et al.*, [34] has reported that *S. cerevisiae* has sophisticated genetic mechanism to regulate cellular iron levels even at a 250 fold excess iron in the media.

Iron is an important co-factor required in several enzymatic processes including for the conserved oxidative phosphorylation and energy (ATP) production [1]. Reduction in iron and heme content in the IDD cells led to reduction in ATP. Dysfunctioning of iron homeostasis also damages structure of cellular organelles and affects normal metabolism [26]. Reduction in the healthy reticulate form of mitochondria in the IDD cells that was observed in the current study may be due to iron deficiency induced damage (Figure **2a**). Iron deficiency or iron overload damages mitochondria and mitochondrial DNA as reported earlier in rat model [35]. However previous studies have not looked at the mitochondrial structure and ATP synthesis in iron deficient conditions. Continuous fusion and fission shape the morphological forms of mitochondrial network and play a role in maintaining the integrity of mitochondria and contribute to healthy survival of the cell [36]. Increase in the clumped mitochondria is an indication of cells entering apoptosis [37]. We have shown that, iron deficiency reduces the proportion of the cells with healthy reticulate mitochondria (Figure **2a**), replenishing iron or 10% PJ supplementation was able to regain and sustain the mitochondrial health. The IDF cells had comparatively lesser percentage of cells with reticulate mitochondria and a higher percentage of cells with fragmented mitochondria (Figure **2a**). Probably, reduction in the mitochondrial integrity could also have contributed the lesser (4.8×10^5 CFU/ml) viable IDF cells when compared to other experimental groups (Table **1**). This shows that, even though, cells can tolerate higher iron content in the media, their physiological status deteriorates. The impact of mitochondrial integrity was

also observed in its ability to synthesize ATP. ATP content in IDP cells was higher when compared to that in IDF and IDN cells (Figure **3b**). However, this was not statistically significant.

Puig *et al*., [38] have reported that metabolic reprogramming gets initiated when there is reduction in the iron concentration of cells. This mechanism helps the cells to survive with altered physiology. Iron deficiency down regulates genes of several mitochondrial metabolic pathways [39]. The decrease observed in the RNA content in the IDP cells and the increased RNA content of IDF cells can be due to the cellular response for iron deficient or iron excess condition (Figure **2b**). We also observed that IDP cells had significantly ($p<0.005$) elevated protein content and lesser lipid content than the IDF and IDN cells (Figure **2c** & **d**). Reasons for this are not clear.

Yeast cells can be a good model to screen interventions to reverse IDA, like plant juices and extracts. PJ has the potential to be of use in managing IDA and it can be further tested in higher organisms. Future studies on the global genome expression and proteomics might give a better understanding of the mechanisms involved in modulation of iron metabolism and cellular physiology by PJ. Scientific exploration of traditional medicines can also provide culturally relevant, sustainable healthcare solutions.

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SUPPLEMENTARY MATERIALS

The supplementary materials can be downloaded from the journal website along with the article.

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