Carbohydrate metabolism modulating effect of *Withania somnifera* in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma is the third leading cause for cancer related death worldwide. Cancer cells exhibit a common set of functional characteristics, i.e. they consume a larger amount of glucose, maintain a much higher rate of glycolysis and convert a majority of glucose into lactic acid even in the presence of oxygen compared to that of normal cells. The high of lactate content in cancer cells (Warburg effect) favors their growth and invasion. The tumor cells preferentially use glycolysis over mitochondrial oxidative phosphorylation for glucose-dependent ATP production. This deviant energetic metabolism is a potential hallmark of cancer cells and has been thought to be the root of tumor formation and growth. In the present study, efficacy of methanolic extract of *Withania sominfera* in carbohydrate metabolism modulating activity is studied in DEN induced hepatocellular carcinoma.

Keywords: Hepatocellular carcinoma, Withania somnifera, N-Notrosodiethylamine (DEN),Carbohydrate metabolism, Warburg effect

1. Introduction

Hepatocellular carcinoma (HCC) currently represents the leading cause of death amongst cirrhotic patients. HCC represents more than 5% of all cancers and the estimated annual number of cases exceeds 500, 000.(El-Serag & Rudolph, 2007). Cancer presents the unique characteristic of a high fermentative glycolytic flux even in the presence of high supply of oxygen, the so-called "Warburg effect" (Ashrafian, 2006; Gatenby & Gillies, 2004). Because of the Warburg effect, tumors produce large amounts of lactate, which confers several advantages for their growth and invasion (Ashrafian, 2006; Gatenby & Gillies, 2004; Marin-Hernandez *et al.,* 2011; Moreno-Sanchez *et al.,* 2009; Semenza, 2008; Sola-Penna, 2008).

The elevated production of lactate has been correlated to the activation of 6-phosphofructo-1-kinase (PFK, phosphofructokinase), the major regulatory glycolytic enzyme. PFK activity has been correlated with the whole glycolytic flux rate and the lactate production rate (Marin-Hernandez *et al.,* 2011; Sola-Penna *et al.,* 2010; Zancan, 2010).

The conversion of highly differentiated glycogenotic hepatocytes via intermediate stages to poorly differentiated cancer cells is usually associated with a degradation of glycogen, a reduction in glycogen metabolizing enzymes and a further increase of the pentose phosphate pathway (Bannasch *et al.,* 1984 & 1997).

N-nitrosodiethylamine (DEN) is a potent hepatotoxic, carcinogen and mutagen (Chuang *et al.,* 2000; Köhle *et al.,* 2008; Sreepriya & Bali, 2006; Tessitore & Bollito, 2006). Human exposure could occur through the diet (meat, whiskey, etc.) (Hecht, 1997; Sen *et al.,* 1980) in certain occupational settings, smoking or with cosmetics, pharmaceutical products and agricultural chemicals (Hecht, 1997).

DEN has been extensively used as an initiating carcinogen in experimental animal models (Chuang *et al.,* 2000; Köhle *et al.,* 2008; Ramakrishnan *et al.,* 2006; Sivaramakrishnan *et al.,* 2008; Sreepriya & Bali, 2005 & 2006; Sundaresan & Subramanian, 2008; Tessitore & Bollito, 2006) and induces hepatic necrosis through metabolic activation by CYP2E1 in experimental animals. Activation of DEN, which takes place mainly in liver microsomes has been shown to stimulate Kupfer cells leading to generate high levels of ROS, capable of damaging liver cells and participating in the induction of hepatocarcinogenesis (Kang *et al.,* 2007).

Withania somnifera is a well-known Indian medicinal plant commonly known as Ashwagandha belonging to the family Solanaceae. It is widely used in many ayurvedic preparations all over the world. Several reports have demonstrated the anti-tumor activity Devi *et al.,*1992, anti-arthritic (Sethi *et al.,*1967), anti-pyretic and anti-inflammatory (Budhiraja, 1987) activity of this plant. The root extract has shown to have immunomodulatory activity (Agarwal *et al.,* 1999), antistress effect Archana & Namasivayam, 1999 and induce iNOS expression (Iuvone *et al.,* 2003).

2. Material and methods

2.1 Sample collection and preparation

Ashwagandha (*Withania somnifera*) was purchased from IMPCOPS, Thiruvanmiyur, Chennai. *Withania Somnifera* was extracted in 95% v/v ethanol in a hermetically closed glass vessel for 4 days at 37°C under occasional shaking. The ethanolic extract was then filtered through a Whatman filter paper #4 and evaporated in a rotary evaporator under reduced pressure at 60° C

2.2 Experimental design

The animals were divided into five groups and each group consisted of six animals.

Group I: Normal control rats fed with standard diet and pure drinking water for 15 weeks

Group II: Rats were induced with hepatocellular carcinoma by providing 0.01% DEN through drinking water for 15 weeks (Ramakrishnan G., *et al*, 2007)

Group III: Hepatocellular carcinoma bearing animals treated with Adriamycin (7.5 mg/kg b.wt. ip) as a positive control (twice in a week) for 2 successive weeks i.e. after the administration of DEN for 13 weeks. (Deepa & Varalakshmi, 2005)

Group IV: Hepatocellular carcinoma bearing animals treated with Withania somnifera alone (500 mg/kg b.wt. ip) for 2 successive weeks (as above)

Group V: Rats treated with Withania somnifera alone for 2 successive weeks (as above)

2.3 Induction of hepatocellular carcinoma

Diethyl nitrosoamine (10mg/dl) was dissolved in water and administered into experimental animals of 130-150 gram body weight through drinking water for 15 weeks. Beginning of 14th week post DEN, treatment was started with Adriamycin (twice in a week, 7.5mg/kg body weight) for 14 days for group III animals. Beginning of 14th week post DEN, treatment was started with *Withania somnifera* (successive days, 500mg/kg body weight) for 14 days for group IV animals.

At the termination of study, animals were sacrificed and the livers were removed. Body weight was recorded periodically from the day of induction till the experimental period was over.

2.4 Withania somnifera administration

Withania somnifera was administered at a dosage of 500mg/kg body weight to a set of DEN- induced tumor bearing animals by oral route. Commencing of 14th week after carcinogen administration, *Withania somnifera* was administered to group IV animals for 14 days.

2.5 Collection of blood and Liver tissue

After the experimental period the animals were anaesthetized with diethyl ether, collection of blood were done through retro orbital procedure and liver tissues were immediately excised, weighed, and processed for homogenization with motor driven Teflon coated homogenizer in ice-cold 0.1M Tris-HCl buffer pH 7.4 to get 10% homogenate. The liver tissue homogenizers were used for following biochemical analysis.

2.6 Measure of Tumor weight

Tumor weight was estimated according to the method of Geren *et al*. (1972). The resultant solid tumor was considered to be prolate ellipsoid with one long axis and two short axis. The two short axis were measured with vernier caliper. The tumor weight was calculated by multiplying the length of the tumor with the square of the width and dividing the product by two.

$$\text{Tumor Weight (g)} = \frac{\text{length (cm) X Width (cm}^2\text{)}}{2}$$

Tumor size obtained from vernier caliper measurements and the actual size measurement of the tumor were found to be nearly the same.

2.7 Estimation of carbohydrate metabolizing enzymes

Hexokinase (ATP:D-hexose-6-phosphotransferase,EC 2.7.1.1): Hexokinase was assayed by the method of Branstrup *et al* (1957).

*Phosphoglucoisomerase: (D glucose-6 phosphate: ketol – isomerase, E.C:5.3.1.9):*This enzyme was assayed by the method of Horrocks *et al* (1963). The enzyme activity was expressed as nmoles of fructose formed / min / mg protein under incubation condi-

tions.

*Aldolase (D-Fructose-1,6-diphosphate:D-glyceraldhyde-3-phosphatelyase E.C:4.1.2.13):*The enzyme was assayed by the method of King (1965c).The enzyme activity was expressed as nmoles of glyceraldehyde formed / min / mg protein under incubation conditions

*Glucose -6- phosphatase (Glucose -6- phosphate: phosphohydrolase, E.C:3.1.3.9):*Glucose -6- phosphatase was assayed according to the method of King (1965.a).The enzyme activity was expressed as nmoles of inorganic phosphorus released / min / mg protein under incubation conditions.

Fructose-1,6-diphosphatase (Fructose -1,: 6- diphosphate phosphohydrolase, E.C:3.1.3.11): Fructose -1,6- diphosphatase was assayed by the method of Gancedo and Gancedo (1971). The enzyme activity was expressed as nmoles of inorganic phosphorus released / min / mg protein under incubation conditions.

2.8 Statistical analysis

For statistical analysis, one-way analysis of variance (ANOVA) was used, followed by the Newman–Keuls multiple comparison test. Mean differences with $P<0.001$, $P<0.01$ and $P < 0.05$ were considered statistically significant.

3. Results

3.1 Tumor and Body weights

Table. 1 represents the effect of Withania somnifera and Adriamycin on the body and liver weight of control and experimental animals. There found to be a significant ($p<0.001$) decrease in the body weight and an increase in the liver weight of the cancer bearing animals (G-II) when compared with control animals (G-1). On treatment with Adriamycin (G-III), there was found to be a significant ($p<0.05$) increase in the body weight and a significantly ($p<0.01$) decrease in the liver weight when compared with the cancer-induced group (G-II). The liver weight was also significantly ($p<0.01$) reduced in Withania somnifera treated HCC bearing animals. However, there found to be no significant difference in the body and liver weights of control animals and the control group treated with a Withania somnifera (G-V).

Table 1. *Effect of Withania Somnifera and Adriamycin on body weight and liver weight in control and experimental animals*

Particulars	Group I (Control)	Group II (DEN induced)	Group III (DEN + ADR)	Group IV (DEN + WS)	Group V (WS alone)
Body weight (gm)	191.04±11.03	147.02±10.01[a]	165.04± 12.02[ab]	169.12± 10.1[abc]	187.12±11.10[NS]
liver weight (gm)	5.64±0.54	7.80± 0.64 [a]	6.13± 0.57 [ab]	5.68± 0.56 [abc]	5.53± 0.52[NS]

Each value is expressed as mean ± S.D. for six male wistar rats in each group.
a: compared with Group I; b: compared with Group II; c: compared with Group III; d: compared with Group IV;
Statistical significance: *p<0.001, @p<0.01, #p<0.05, NS-Not significant

3.2 Carbohydrate metabolizing enzymes

Fig. 1 - 2 show the activity of glycolytic and gluneogenic enzymes in liver of control and experimental animals. The activity of hexose, phosphoglucoisomerase and aldolase were found to be appreciably ($p<0.001$) increased, where as the activities of glucose-6- phosphatase and fructose-1,6-diphosphatase were significantly ($p<0.001$) suppressed when compared with control animals. Treatment with Adriamycin in group III ($p<0.01$) and Withania Somnifera group IV ($p<0.01$) HCC bearing animals, the levels of glycolytic enzymes were significantly decreased when compared to untreated group II HCC bearing animals. On treatment with *Withania somnifera* significantly ($p<0.001$) brought back to these enzymes activities in group I animals to near normal levels.

4. Discussion

A common set of functional characteristics of cancer cells is that cancer cells consume a large amount of glucose, maintain high rate of glycolysis and convert a majority of glucose into lactic acid even in the presence of oxygen compared to that of normal cells

(Warburg's Effects). In addition, cancer cells exhibit substantial alterations in several energy metabolism pathways including glucose transport, tricarboxylic acid (TCA) cycle, glutaminolysis, mitochondrial respiratory chain oxidative phosphorylation and pentose phosphate pathway (PPP).

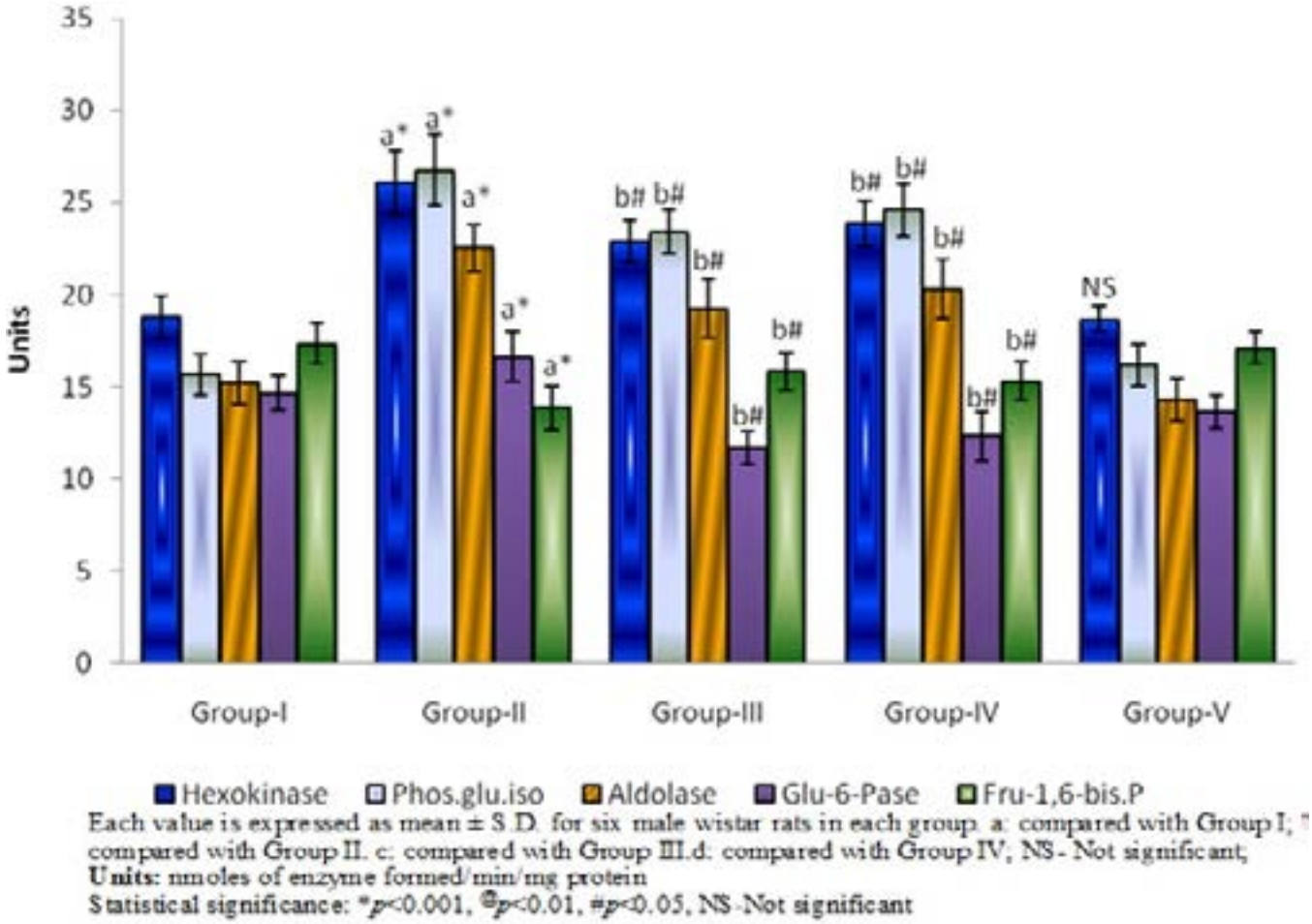
Since cancer cells rely on anaerobic metabolism to produce a variable but generally significant portion of their energy requirements, inhibition of the glycolytic pathway is an obvious approach that may exploit the high glucose consumption by cancer cells. Indeed, there is clear evidence that inhibition of glcyolysis can result in cancer cell death particularly in hypoxic environment due to ATP depletion (Jones *et al.,* 2005).

Development of different types of tumors is accompanied by characteristic alteration in the activities of enzymes, particularly those involved in carbohydrate metabolism (Ahn *et al.,* 1992). The growth rate of hepatomas and their glycolytic enzymes activities are significantly correlated (Royds *et al.,* 1987). Transformation of many cell types are accompanied by an increased in glucose catabolism, and transformed cells generally have raised activities of key enzymes (Baggetto, 1992).

Decreased activities of glucose-6-phosphatase and fructose-1,6-diphosphatase reveal the progressive failure of gluconeogenesis in liver tumors(Baggetto,1992).

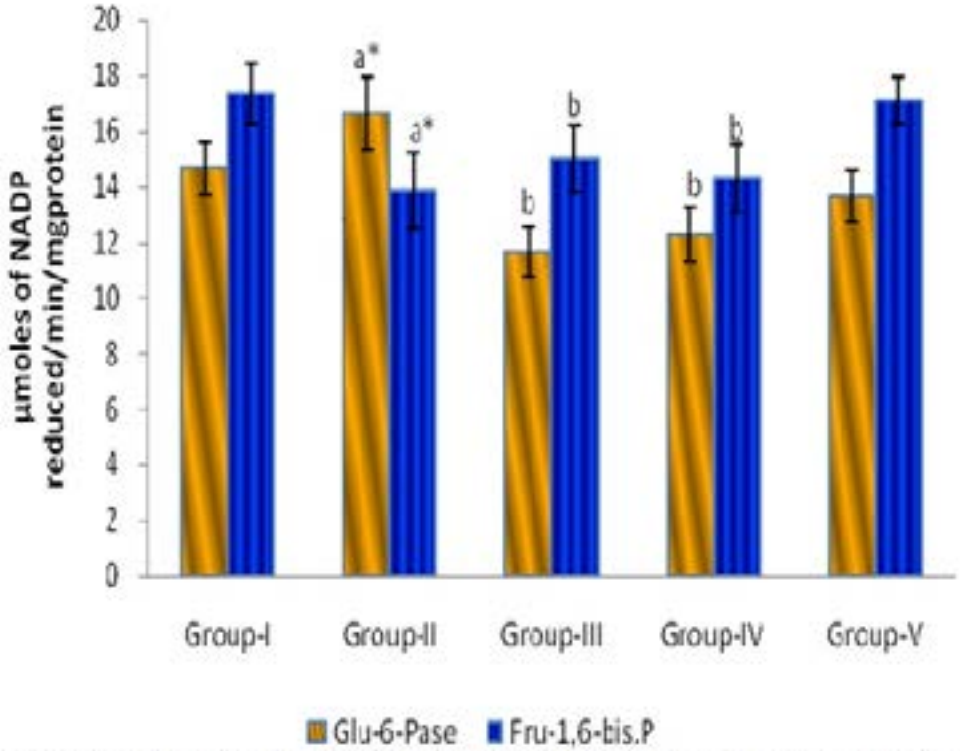
The data presented in Fig.1-2 reveal a profound influence of *Withania somnifera* administration on enzymes of energy metabolism in rat liver, which is manifested by changes in the activity or content of numerous key enzymes of different metabolic pathways, *e.g.,* glycolysis and gluconeogenesis. A significant increased in glycolytic enzymes such as hexokinase, phosphoglucoisomerase and aldolase was observed in DEN and Adriamycin treated HCC bearing animals. The observed increased might be due to an inhibition in the transport of glucose by carcinogen.

Fig.1. *The activity of carbohydrate metabolizing enzymes in the liver of Control and experimental animals*



Each value is expressed as mean ± S.D. for six male wistar rats in each group. a: compared with Group I; b: compared with Group II. c: compared with Group III.d: compared with Group IV, NS - Not significant;
Units: nmoles of enzyme formed/min/mg protein
Statistical significance: *p<0.001, @p<0.01, #p<0.05, NS -Not significant

Fig.2. *The activity of Glu-6- PDH and fru-1, 2 bisphosphatase in the liver of Control and experimental animals*



Each value is expressed as mean ± S.D. for six male wistar rats in each group. a: compared with Group I; b: compared with Group II. c: compared with Group III. d: compared with Group IV;
Statistical significance: *p<0.001, @p<0.01, #p<0.05, NS-Not significant

5. Conclusion

Our findings indicate that administration of Withania Somnifera and Adriamycin significantly reduced the alteration induced by DEN in glycolytic and gluconeogenic enzymes. However, withania Somnifera at the concentration used did not affect the glycolytic and gluconeogenic enzyme activities of the control animals. The selective action of *Withania Somnifera* on energy metabolism in liver cancer may account for its inhibitiory effect on cancer cells.

6. References

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