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Effectiveness of combined treatment using *Spirulina* and vitamin A against chronic arsenicosis in rats

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Chronic arsenicosis remains a condition of public health concerns in many countries of the world and has been linked to many other diseases. This condition has been managed in humans using a combination of therapy with differing outcomes. We conducted a controlled experiment to assess the effect of chronic arsenicosis on hematological and biochemical changes in Long-Evans rats and to assess the protective role of *Spirulina* combined with vitamin A following experimental arsenicosis, using daily oral doses of sodium arsenite for 63 days. The values of SGOT (Serum glutamate oxaloacetate transaminase) and SC (Serum creatinine) increased significantly ($P<0.01$) in all the treated groups of rats (T_1, T_2, T_3 and T_4) compared to the control (T_0) group, but *Spirulina* combined with Vitamin A produced values significantly comparable to the untreated control group. Whereas SGPT (Serum glutamate pyruvate transaminase) showed slight significance differences among the treatment groups, *Spirulina* combined with Vitamin A appeared most effective in managing arsenic treatment. *Spirulina* + Vitamin A increased the values of TEC, TLC and Hb (Total erythrocyte count, Total leukocyte count and Hemoglobin) against arsenic toxicity in rats but showed no significance differences. In conclusion, the combination of *Spirulina* and vitamin A were found more effective in the prevention of chronic arsenicosis in rat than using these substances (*Spirulina* or Vitamin A) alone.

Key words: Chronic arsenicosis, hemato-biochemical changes, *Spirulina*, Vitamin A, transaminases.

INTRODUCTION

Arsenic-related diseases remain a problem of serious public health concerns in many countries including Argentina, Bangladesh, Chile, China, India, Mexico, Thailand and the USA (Smith et al., 2000; Khalequzzaman et al., 2005; WHO, 2011). Arsenicosis has been linked to heart disease and hypertension (Tseng et al., 2003), cancer (Smith et al., 1992), stroke (Chiou et al., 1997), cerebro-vascular diseases, chronic lower respiratory diseases (Hendryx, 2009) and diabetes (Navas-Acien et al., 2008; Kile and Christiani, 2008). Chronic arsenic poisoning results from drinking contaminated well water over a long period of time and the World Health Organization recommends a limit of 0.01 mg/L (10 ppb) of arsenic in drinking water, though this limit also can predispose to arsenicosis (WHO, 2001; Walker and Fosbury, 2009; Prozialeck et al., 2008). More recent findings show that consumption of water with levels as low as 0.00017 mg/L (0.17 ppb) over long period of time can still lead to arsenicosis (WHO, 2001). *Spirulina* is an algae product that has been used in human and animal food supplements. It is made from the cyanobacteria *Arthrospiraplatensis* and *Arthrospiramaxima.*

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These plants are widely cultivated especially in regions where arsenicosis remains of huge importance in public health (Vonshak, 1997). *Spirulina* has been proved to have inhibitory effect on HIV in certain human cells (Ayehunie et al., 1998), and when used alone or in combination with vitamins and/or minerals was found to be effective in the removal of arsenic from arsenic-loaded tissues in various species including man (Misbahuddin et al., 2006; Awal, 2007). It is also used as oral supplementation with vitamin A (retinol) in the treatment of cutaneous arsenicosis as recorded by Hall (1946) and Ahmad et al. (1998).

Arsenicosis presents with significant changes in the serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum creatinine, urea, uric acid levels and various hematological parameters like TEC, TLC, Hb, blood sugar level in the Swiss albino rats (Yasmin et al., 2011). Acute, intermediate or chronic exposure to arsenic similarly resulted in the development of anemia and leucopeniain in a previous study (Flora et al., 2007). This study was undertaken to determine the effect(s) of *Spirulina* and vitamin A, including their combination on some hematological and biochemical parameters in Long-Evans rats with experimentally-induced chronic arsenicosis.

MATERIALS AND METHODS

Preparation of sodium arsenite solution

On the basis of the mean total body weight of the rats, a daily dose of 4 mg/kg bodyweight of sodium arsenite ($NaAsO_2$) was weighed out and administered to each group via the drinking water. To ensure that the daily dosage was taken, 10 ml drinking water per group was initially allotted for mixing $NaAsO_2$ and only after the drinker is completely empty was fresh water served *ad libitum.*

Preparation of *Spirulina* powder

Each tablet of *Spirulina* (containing 500 mg of *Spirulina platensis*) was made to a homogeneous powder with the help of pestle and mortar. Then the required amount of *Spirulina* was measured with the help of electric balance. The powdered *Spirulina* was kept in desiccators to prevent water absorption and change in quality of the product. For proper homogeneity, small amount (≈15 ml) of distill water was added to make a suspension and then the suspension was added drop by drop to the feed with the help of a small stainless steel spoon and simultaneously the feed was stirred by a glass rod for homogenous mixing. After completion of the mixing, the mixed feed was dried in an electric oven at 50ºC for 24 h and kept in air-tight plastic container.

Preparation of vitamin A

Each vitamin A capsule contained 50,000 I.U of vitamin A (Retinol Forte Capsule, 2500 IU/kg feed) was mixed with 2 kg dried pellet feed. For proper mixing small amount (≈10-15 ml) of distill water was added to make an emulsion and the emulsion was added drop by drop to the feed with the help of a small stainless steel spoon and simultaneously the feed was stirred by a glass rod for homogenous mixing. After completion of the mixing, the mixed feed was dried and kept in air-tight plastic container.

Assignment of subjects to groups and feed

Sixty Long-Evans rats were purchased for use in this study. They were conditioned to the new environment and given feed and water *ad-libitum* prior to treatment. Each rat was weighed and the sixty animals were randomly divided into 5 groups of 12 rats each viz: control group (T_0), Arsenic (As) treated group (T_1), Arsenic + *Spirulina* treated group (T_2), Arsenic + Vitamin A treated group (T_3) and Arsenic + *Spirulina* + Vitamin A treated group (T_4). Rats in Group T_0 were given feed *ad-libitum* and water. Rats in Groups T_1, T_2, T_3 and T_4 were treated orally with 4 mg of sodium arsenite/kg body weight daily, for 63 days ($NaAs_2O_2$, MW 197.84 g/mol, May & Baker Ltd, Dagenham, England). In addition to the sodium arsenite treatment, the rats in groups T_2 and T_4 were simultaneously fed with *Spirulina* (Life Line International Company, Bangladesh) at 1 g/kg of feed *ad-libitum* while groups T_3 and T_4 were fed vitamin A (Retinol Forte®; Drug International Limited; Bangladesh) at 2500 IU/kg of feed *ad-libitum* up to 63 days respectively. All animals were fed *ad-libitum* throughout the period of the experiment.

Sampling

Starting from day 21 post-treatment, and every 21 days thereafter, 4 rats from each group were anesthetized using chloroform and approximately five milliliters (ml) of blood was collected directly from heart of each rat by using sterile syringe. The blood from each rat was then transferred into three tubes each. For the biochemical test, blood was taken into pre-marked centrifuge glass test tubes immediately after collection and was kept at room temperature for 1 h without agitation to clot with a view to collect serum. The harvested sera were kept at-20°C until used. For the hematological parameter test and arsenic determination in blood, 1 ml of each blood was taken separately into 2 EDTA coated tubes. Bloods samples for hematological investigation were preserved at +4°C and those for arsenic level in blood at -20°C until tested. All blood samples were taken on day 21, day 42 and day 63.

Biochemical tests

Sera were thawed on the laboratory bench (≈+25°C) and the SGOT, SGPT activity and serum creatinine were determined through the use of Reflotron® Plus (Boehringer Mannheim, Germany) according to the method described by Deneke and Rittersdorf (1984) and Deneke et al. (1985).

Determination of hematological parameters

Total erythrocyte count (TEC), total leukocyte count (TLC) and hemoglobin concentration (Hb) were determined using the method described by Lamberg and Rothstein (1977).

Statistical analysis

Since the experimental data were designed following the complete randomized design (CRD), it was statistically analyzed using one-way analysis of variance (ANOVA) and SPSS v13 software. Mean comparisons of the treatments were made by the Duncan's Multiple Range Test (DMRT) (Steel and Torrie, 1980).

Table 1. Effects of different treatment on biochemical parameters (SGOT, SGPT and serum creatinine values) in rats.

Treatment	Biochemical parameter		
	SGOT (U/L)		
	Day 21	Day 42	Day 63
Control (T_0)	231.67±0.88^{c}	235.67±2.40^{c}	232.00±3.79^{e}
Arsenic (T_1)	322.33±2.03^{a}	381.67±2.91^{a}	402.00±0.001^{a}
Spirulina+ Arsenic (T_2)	294.33±6.36^{b}	281.00±1.73^{b}	282.00±5.57^{c}
Arsenic + Vit A (T_3)	315.00±5.86^{a}	312.67±2.33^{a}	306.67±5.55^{b}
Arsenic+ *Spirulina*+Vit A (T_4)	241.00±1.15^{c}	239.33±1.86^{c}	247.67±2.03^{d}
Level of significance	P<0.01	P<0.01	P<0.01
		SGPT (U/L)	
Control (T_0)	41.53±1.17^{c}	41.97±1.45^{c}	42.30±1.14
Arsenic (T_1)	58.90±2.29^{a}	49.73±1.03^{a}	49.03±1.70
Spirulina+ Arsenic (T_2)	44.53±1.17^{b}	46.67±1.47^{b}	44.47±2.18
Arsenic + Vit A (T_3)	46.30±1.04^{b}	47.83±2.07^{b}	44.57±1.07
Arsenic+ *Spirulina*+Vit A (T_4)	44.20±1.61^{b}	42.70±1.89^{c}	43.28±1.12
Level of significance	P<0.01	P<0.01	NS
		Serum creatinine (mg/dl)	
Control (T_0)	0.51±0.001^{b}	0.51±0.001^{b}	0.51±0.001^{b}
Arsenic (T_1)	0.61±0.05^{a}	0.64±0.02^{a}	0.67±0.04^{a}
Spirulina+ Arsenic (T_2)	0.52±0.001^{b}	0.56±0.001^{ab}	0.51±0.001^{b}
Arsenic + Vit A (T_3)	0.54±0.01^{ab}	0.62±0.05^{c}	0.61±0.01^{c}
Arsenic+ *Spirulina*+Vit A (T_4)	0.52±0.001^{bs}	0.51±0.01^{b}	0.51±0.001^{b}
Level of significance	P<0.05	P<0.01	P<0.01

Values indicate the mean±SE; NS = no significant difference between values; Values with similar superscripts did not differ significantly; Values with dissimilar superscripts differed significantly. Evaluation was done using Duncan multiple range test (DMRT).

RESULTS

Biochemical parameters

Serum glutamate oxaloacetate transaminase activity *(SGOT)*

The values of SGOT on day 21 ranged between 231.67 to 322.33U/L and these values increased to 235.67 to 381.67U/L and 232.00 to 402.00U/L by days 42 and 63 respectively (Table 1). The highest values were observed in the arsenic only group (T_1) while the lowest values were observed in the control group. There were significant differences within the groups during the three days (21, 42 and 63) of measurement (P<0.01). It appears that while *Spirulina* alone has some effect in lowering the SGOT values in response to prolonged administration of arsenic, the combination of *Spirulina* and Vitamin A produced a more significant reduction in SGOT level comparable to the control group (P<0.01, Table 1). Vitamin A administration alone did not significantly reduce the effect of arsenicosis on SGOT level. Overall, chronic arsenicosis significantly increased the level of SGOT in the blood by up to 39.13% (90.66U/L) to 73.28% (402.00U/L; Table 1).

Serum glutamate pyruvate transaminase activity *(SGPT)*

Continuous administration of arsenic to Long-Evans rats caused a significant increase in the blood SGPT level to between 15.91% (6.73U/L) and 41.83% (17.37U/L). There was a huge surge in values of SGPT on day 21 in response to arsenic administration but these values reduced slightly over the next 42 days (Table 1). The highest values of SGPT were observed in the arsenic only group (T_1) while the lowest values were observed in the control group. There were significant differences within the groups during days 21 and 42 but this difference became insignificant by day 63 (Table 1). It is probable that prolonged administration of arsenic will be followed in a long term by apparently normal levels of SGPT (P<0.01). Though both *Spirulina* and vitamin A (individually or in combination) reduced the level of SGPT in response to arsenic administration, there was no significant difference between the treatments.

On day 42, the values of SGPT was the highest in T_1 group rats and lowest in control group and the As values were significantly (P<0.01) increased in T_1, T_2 and T_3 group rats compared to control group rats. But the As values of T_4 group rats were not significant compared to

control group rats. The values in T_2 and T_3 were statistically significantly different ($P<0.01$) compared to T_1 and T_4 group rats and the difference in As content between T_2 and T_3 group rats were not significant. However the As contents increased in T_2 and T_3 group but decreased in T_1 and T_4 groups on day 42 compared to day 21. On day 63 the SGPT values were highest in T_1 and the lowest in T_0 group rats but the difference were statistically not significant among themselves. However the As contents increased in T_4 group but decreased in T_1, T_2 and T_3 groups on day 63 compared to day 42 (Table 1).

Serum creatinine

On day 21 the values of serum creatinine were observed highest in T_1 and the lowest in T_0 group rats. The differences were found significant ($P<0.05$) on day 21. The differences of As values of T_2, T_3 and T_4 group rats were not significant compared to control group but the increase observed in T_1 group rats was statistically significant compared to control group rats. And the values in T_2, T_3 and T_4 group rats were statistically significantly different ($P<0.05$) compared to T_1 group rats and the difference in As content among T_2, T_3 and T_4 group rats was not significant.

On day 42, the values of serum creatinine were the highest in T_1 group rats and lowest in control group. The differences were found significant ($P<0.01$) on day 42 (Table 1).

The As values were sharply and significantly ($P<0.01$) increased in T_1 and T_3 group rats compared to control group rats but the As values of T_2 and T_4 group rats were not significantly different compared to control group. The difference observed among T_1, T_3 and T_4 groups was statistically significant ($P<0.01$).. Again the values of As content in T_1 group were not significantly different compared to T_2 group rats; similarly there was no significant difference between T_4 group and T_2 group rats. The differences of As content between T_2 and T_3 were statistically significant ($P<0.01$). However the As contents increased in T_1, T_2 and T_3 group but decreased in T_4, groups on day 42 compared to day 21. On day 63, the values of serum creatinine was the highest in T_1 group rats and lowest in control group. The differences were found significant ($P<0.01$) on day 63. The As values sharply and significantly ($P<0.01$) increased in T_1 and T_3 group rats compared to control group but the As values of T_2 and T_4 group rats were not significant compared to control group while the values in T_2 and T_4 were statistically significant ($P<0.01$) compared to T_1 and T_3 group rats. Also the differences in As content between T_2 and T_4 group rats were not significant but the difference in As content between T_1 and T_3 group rats were significant on day 63. However the As contents increased in T_1 group but decreased in T_2 and T_3 groups on day 63 compared to day 42.

Hematological parameters

Total erythrocyte count (TEC)

Total erythrocyte counts on day 21 was found highest in T_0 group rats and lowest in T_1 group rats but the differences were not statistically significant among all groups of rats.

On day 42, total erythrocyte count was found highest in T_0 and lowest in T_1 group of rats. The differences were found significant ($P<0.05$). The As values of T_2, T_3 and T_4 group rats were statistically non significant compared to control group but the As values of T_1 group rats were statistically significant compared to control group. The differences of As value among T_1, T_2, T_3 and T_4 group rats were statistically non significant. However the As contents increased in T_2, T_3 and T_4 group but decreased in T_1 groups on day 42 compared to day 21. Total erythrocyte counts on day 63 was found highest in control group rats and lowest in T_1 group rats but the difference were not statistically significant among all group of rats. However the As contents increased in all treated group on day 63 compared to day 42 (Table 2).

Total leukocyte count (TLC)

Total leukocyte counts on day 21 was found highest in control group rats and lowest in T_1 group rats but the difference were not statistically significant among all group of rats. But the values gradually increased by 42 days compared to day 21 value.

On day 42, total leukocyte counts was found highest in control group rats and lowest in T_1 group rats but the differences were not statistically significant among all group of rats. However the As contents were increased in all treated groups on day 42 compared to day 21. Total leukocyte counts on day 63 was found highest in control group rats and lowest in T_1 group rats but the differences were not statistically significant among all group of rats. However the As contents decreased in all treated groups on day 63 compared to day 42 (Table 2).

Hemoglobin concentration (Hb)

Total Hemoglobin concentration (Hb) on day 21 was found highest in control group rats and lowest in T_1 group rats but the differences were not statistically significant among all groups of rats.

On day 42, total hemoglobin concentration (Hb) was found highest in control group rats and lowest in T_1 group rats but the differences were not statistically significant among all group of rats. However the As contents increased

Table 2. Effects of different treatment on haematological parameters (Total erythrocyte counts, total leukocyte counts and hemoglobin concentration) in rats.

Treatment	Biochemical parameter		
	Total erythrocyte count TEC (million/µl)		
	Day 21	Day 42	Day 63
Control (T_0)	7.40±0.36	8.52±0.22[a]	8.97±0.73
Arsenic (T_1)	6.77±0.14	5.99±0.56[b]	7.23±0.40
Spirulina+ Arsenic (T_2)	7.05±0.35	7.56±0.57[ab]	7.78±0.20
Arsenic + Vit A (T_3)	6.83±0.15	7.51±0.44[ab]	7.57±0.36
Arsenic+ *Spirulina*+Vit A (T_4)	7.29±0.29	7.64±0.37[ab]	8.22±0.27
Level of significance	NS	P<0.05	NS
	Total leukocyte count (TLC) (thousand/µl) values in rats		
Control (T_0)	9.57±0.28	10.88±0.54	10.82±0.64
Arsenic (T_1)	9.09±0.47	10.24±0.24	9.79±0.56
Spirulina+ Arsenic (T_2)	9.40±0.35	10.41±0.51	9.88±0.62
Arsenic + Vit A (T_3)	9.29±0.34	10.26±0.23	9.87±0.63
Arsenic+ *Spirulina*+Vit A (T_4)	9.56±0.76	10.76±0.86	10.72±0.66
Level of significance	NS	NS	NS
	Hemoglobin concentration (Hb) (gm/dl) values in rats		
Control (T_0)	10.47±0.71	11.27±0.52	11.82±0.13
Arsenic (T_1)	9.80±0.35	9.54±0.44	10.20±0.50
Spirulina+ Arsenic (T_2)	10.17±0.39	10.54±0.75	11.13±0.53
Arsenic + Vit A (T_3)	10.00±0.40	10.53±0.77	10.73±0.58
Arsenic+ *Spirulina*+Vit A (T_4)	10.27±0.35	10.80±0.72	11.73±0.81
Level of significance	NS	NS	NS

Values indicate the mean±SE; NS = no significant difference between values; Values with similar superscripts did not differ significantly; Values with dissimilar superscripts differed significantly. Evaluation was done using Duncan multiple range test (DMRT).

in T_2, T_3 and T_4 groups but decreased in T_1 groups on day 42 compared to day 21. Total Hemoglobin concentration (Hb) on day 63 was found highest in control group rats and lowest in T_1 group rats but the differences were not statistically significant among all groups of rats. The As contents increased in all treated group on day 63 compared to day 42 (Table 2).

DISCUSSION

In this study, the values of SGOT (Table 1) were increased significantly (P<0.01) in the blood samples of the treated groups of rats (T_1, T_2, T_3 and T_4) compared to control (T_0). Although this finding disagreed with the previous findings that SGOT was reduced by As alone (Mahaffey et al., 1981), it concurred with the findings of Yasmin et al. (2011) who indicated similar results. In *Spirulina* treated (T_2), Vitamin A treated (T_3) and *Spirulina* plus Vitamin A treated (T_4) experimental arsenicosis groups, there were significantly decreased values of arsenic recorded (P<0.01) compared with the arsenic treated group of rats (T_1). Since arsenic toxicity can cause hepatic insufficiency and *Spirulina* and Vitamin A treatment improved the hepatic functions apart from decreasing the level of arsenic in blood, it can be concluded that *Spirulina* improve liver function by reducing hepatic damage due to heavy metal exposure and drug abuse (González et al., 1999).

Although the levels of SGPT in serum differ significantly at day 21 and day 42 (P<0.01), it normalized in all groups and there was no significant difference by day 63 except for the arsenic group (Table 1). It is possible that individual treatment using Vitamin A or *Spirulina* or a combination of the two will not produce a significantly different result in long term arsenicosis. Interestingly, the level of SGPT did not change drastically in arsenic treated group (T_1) between days 42 and 63. It may be that once a peak level of arsenicosis is reached, the SGPT level will adjust to it and maintain a peak value. Kaur et al. (2005) had earlier found that no change was observed in SGPT level associated with arsenicosis over a 90-day period. However, our result differed partly with this report as the 21 day collection of serum in our study revealed peak SGPT levels in arsenic treated group. Yasmin et al. (2011) had also recently reported a 16.67% increase in SGPT level of arsenic treated mice as compared to the control.

There were significant differences in serum creatinine levels between the control group and all other treatment groups throughout the study period (Table 1). This disagreed with the findings of Nabi et al. (2005) in human being which showed that patients of arsenicosis had significantly lower level of serum creatinine compared to the control. It is possible that the differences observed in these two studies are related to the differences in species used for the study. Zhang et al. (1995) had also observed that there is a relationship between arsenic level and degree of chronic renal insufficiency in men. The result of this study was dissimilar with the findings of Yasmin et al. (2011) Islam et al. (2009) and Roger et al. (2000) which concluded that there were no significant rises in the serum creatinine levels of arsenic treated mice. A slight reduction in serum creatinine was however observed with the time progression in our study for the *Spirulina* and Vitamin A treated group (T_4), suggesting that *Spirulina* and Vitamin A may improve liver function.

Our findings also revealed that the TEC in the T_1 group was lower compared to control and other treatment groups. Whether this observation is due to relative or absolute anaemia cannot be established in this study. We however confirmed that treatment with vitamin A and *Spirulina* either alone or in combination improved the TEC, although the combination produced a better result. Chronic arsenic toxicity might cause decreased in TEC and anaemia. The effects observed in this study and outcomes of treatment using *Spirulina* plus vitamin A treatment had earlier been corroborated by Gupta et al. (2007); Breton et al. (2006) and Juruli and Katsitadze (2007). They reported decreasing RBC level with increased concentration of arsenic due to arsenic metabolism and its methylating activity.

There was no significant difference in TLC and Hb values observed among all the groups during the entire study period but slight increase in TLC and Hb values (Table 2) in rats of all other treated groups (T_2, T_3 and T_4) compared to arsenic treated group (T_1) was observed. The result is similar to the findings of Yasmin et al. (2011); but contradicts that of Rousselot et al. (2004) where they found decreased WBC level and constant Hb level when mice were given higher dose of arsenic and that might be due to apoptotic effect of arsenic on plasma cells. It is possible that since arsenicosis is not an infectious condition, there was no mobilization of the physiologic system to increase the production of white blood cells or lymphocytes. Whether *Spirulina* and vitamin A had an influence on the values of TLC and Hb against arsenic toxicity in rats cannot be established in this study. A more carefully planned research targeting this objective is required to be undertaken.

In conclusion, though we have established the ameliorative effect of *Spirulina* and vitamin A on arsenocisis, it will be desirable to conduct a long term study since this condition is often a chronic situation and a three month study like ours cannot be extrapolated for a long term life threatening condition of arsenic toxicities.

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