TOXICOLOGY AND FREE RADICALS SCAVENGING PROPERTY OF TAMRA BHASMA

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ABSTRACT

Free radicals are implicated in various chronic diseases. There has always been a search for new antioxidants. In this paper we have investigated Tamra bhasma, a metallic ayurvedic preparation. It is a time-tested medicine in Ayurveda and is in clinical use for various ailments specifically the free radical mediated diseases. Our results show that Tamra bhasma inhibits lipid peroxidation (LPO), prevents the rate of aerial oxidation of reduced glutathione (GSH) content and induces the activity of superoxide dismutase (SOD) in rat liver homogenate in the bi-phasic manner. The drug was orally given for 7, 15 and 30 days in different doses. Best protective response was found at the dose of 0.5mg/100g body weight in albino rats, although it showed some histopathological changes at the dose of 20mg/100g body weight. The results suggest that this Ayurvedic preparation is not merely a source of copper metal, but it is a strong anti-oxidant with no detectable adverse effect in lower doses of therapeutic range.

KEY WORDS

Copper, antioxidant, superoxide dismutase, Tamra bhasma, Ayurvedic metallic preparation, antilipid peroxidation, toxicology of metals

INTRODUCTION

Free radicals are spontaneously produced in the mitochondria as a byproduct of electron transport chain. An enzyme called superoxide dismutase is responsible for its quick removal by its dismutation to hydrogen peroxide, which is finally hydrolyzed to water and oxygen with the help of catalase and glutathione peroxidase. In old age, the rate of production of free radical predominates than its removal, leading to peroxidation of all macromolecules including lipids, proteins and nucleic acids. Besides, many metabolic disorders such as diabetes, atherosclerosis, arthritis, nervous diseases etc are now established free radical mediated diseases.

The antioxidants are in use for the management of these diseases. Some of them are directly inactivating or quenching these free radicals like

fat soluble vitamins, glutathione and various phytochemicals and others are responsible for the activation of endogenous antioxidant enzymes like selenium containing glutathione peroxidase and copper superoxide dismutase respectively. Copper is a trace element, involved as an essential constituent of certain enzymes such as catalase, tyrosinase, serum oxidase, ascorbic acid oxidase, cytochrome oxidase, etc(1). Besides, copper is also important for the absorption of iron through the intestine. It is also reported for the management of leucoderma.

In the Ayurvedic description, several metallic preparations are in clinical use since 12th century AD. They have specific methods for their detoxification and bhasma preparation, which becomes suitable for clinical use in therapeutic doses. Since these preparations are sustaining themselves since centuries in clinical use, therefore one can not exclude its use just by saying that heavy metals are toxic. Proper documentation is the demand of time to validate the claims about these metallic preparations. Copper is one such metal. In the Ayurvedic system of medicine, Tamra bhasma is recommended in the dose of 10 mg to 30 mg for an adult (70 kg body weight; 0.2mg/kg) to manage liver disorders, gastro-intestinal tract (GIT)

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disorders, old age diseases, leucoderma, cardiac problems (2) and various other free radical mediated disorders (3), either alone or as herbo-mineral compositions. Deficiency of copper in the body causes weight loss, bone disorders, microcytic hypochromic anaemia, hypopigmentation, graying of hair and demyelination of nerves etc (4).

We have earlier reported its *in vitro* response on lipid peroxidation (5). Here, an *in vivo* study of *Tamra bhasma* has been made in albino rats on several free radical species and various toxicological parameters. The dose range was decided on the basis of human dose that is 10- 30 mg/70 kg adult body weight. For toxicological study, dose was chosen on higher side with an objective to ascertain its safety margin.

MATERIALS AND METHODS

Chemicals – Copper turnings (99.9% pure), thiobarbituric acid, glacial acetic acid, EDTA, sodium dodecyl sulphate (SDS), hydrochloric acid (HCl), nitro blue tetrazolium (NBT), methionine, riboflavin, phosphotungstic acid (PTA) were purchased from Central Drug House (Pvt.) Ltd. Reduced glutathione and DTNB (5,5' Dithio-bis (2- nitrobenzoic acid) were procured from Sigma Chemical Co, St. Louis, MO, USA. Albino rats of inbred Charles Foster strain, (body weight between 100 to 150 g) were arranged from Central Animal House of our Institute.

Preparation of Tamra bhasma

Tamra bhasma was prepared by standard method described in the Ayurvedic classical text(2). Process of preparation was divided into 2 parts, *Sodhan* (purification) of raw copper and bhasma preparation. For purification, copper turnings were repeatedly heated to red hot condition and immediately quenched in different cold solvents in a sequence of *Sessamum* oil, butter milk, cow urine, sour gruel, decoction of *Dolichos biflorus* and juice of *Oxalis omeiculatum*. This process was repeated for 5 times in each solvent. The end product of this process is claimed as *sodhit* copper (purified copper). It is brittle in nature and black in colour.

For the bhasma preparation, equal quantity of purified copper, purified sulphur (*Shodhit gandhak*) and purified mercury (*Shodhit parad*) were mixed. In the beginning, mercury and copper were titrated with lemon juice to make the amalgam. Then equal quantity of sulphur was added to it and again titrated till black fine powder was formed. This powder was

heated up to 700° C for 9 hrs in a *kupi yantra* (glass bottle wrapped with seven layers of mud smeared cotton cloth) by gradual rise in temperature in a thermostatic controlled muffle furnace. Next day Tamra bhasma was collected from the bottom of the *kupi* and fine powder was made which passed 100% through 200 mesh sieves. This was tested for its quality as per classical methods (2). Bhasma was found floating on water surface. It did not change the colour of curd, when kept in contact for 24 hrs. It was not shining as the original metal, in the sunlight. It was further characterized by X ray defraction spectrum. It showed a peak of copper sulphide of intensity 27 and spacing value of 1.87. It also showed 4 extra peaks at different intensity and spacing value, which were not reported in the literature. The data was similar to what has been reported, previously by this laboratory (6).

Determination of optimum dose

Animals were randomly selected and housed in plastic bottom cages. They were allowed free access to standard laboratory chow (Hindustan Lever, Mumbai) and water and kept on overnight fast before sacrifice. The Institute animal care and use committee approved the protocol of this study. To determine the LD₅₀ and optimum dose of Tamra bhasma in our experimental conditions, rats were divided in different groups (n=6) as shown in Table 1. Animals of the first group received 1ml drug vehicle (20% gum acasia in distilled water) and served as sham control. The other group daily received Tamra bhasma suspension in different doses up to 30 days. The survival of each animal was monitored through out the experiment. At the end, blood was collected to assay the liver and kidney function in terms of serum GOT, GPT, alkaline phosphatase, lactate dehydrogenase, serum urea and serum creatinine. Finally the animals were sacrificed by cervical dislocation to collect liver and kidney for histological studies by using standard protocol(7). All the above estimations were carried out by using standard commercial kits.

Toxicological study

This study was divided into 3 phases (a) acute study for 3 days (b) sub-acute studies for 15 days and (c) chronic studies for 90 days. In these studies different doses of Tamra bhasma were orally given to rats as shown in Table 2. In all the experiments hepatocellular enzymes namely SGOT, SGPT, alkaline phosphatase, LDH and two metabolites for

the kidney function tests namely serum urea and serum creatinine were estimated. Histopathological changes in liver and kidney were carried out by using paraffin wax block and staining with hematoxylin and eosin. Inverse relation in the dose and duration of drug administration was maintained because in normal course of treatment, Tamra bhasma was given to the patients in very low dose and also for shorter duration.

Effect on antioxidant enzymes

Albino rats were divided into different groups (n = 6) as indicated in the respective tables. In the first group, which served as sham control, only drug vector (20% gum-acasia in water) was orally given in the dose of 1ml/100g body weight. In the treated groups, different concentrations of the *Tamra bhasma* were given for different duration that is 7, 10 and 30 days. Overnight fasted rats were anaesthetized by intra-peritoneal injection of pentobarbitone (3mg/100g body weight). Liver was visualized, perfused with normal saline to make it blood free and dissected out. A small portion was fixed in 10% Bouin's solution for histopathological studies. Further 10% liver homogenate (0.8 - 1.5 mg protein in 0.1 ml) was separately prepared in a glass-teflon homogenizer, for the estimation of TBARS and reduced glutathione (GSH). Protein concentration was measured by using standard method of Lowry *et al* (8). To monitor the activity of superoxide dismutase, the homogenate was subjected to one step purification by differential centrifugation in a refrigerated centrifuge at 12,000 rpm for 15 minutes. Rate of aerial oxidation of reduced glutathione was also estimated. For this, 3 ml of 10% liver homogenate was kept in a 35 mm petri dish and after every 10 minutes, 250 μ l of the homogenate was taken out to estimate the concentration of reduced GSH (9).

Estimation of Thiobarbituric acid reactive substances (TBARS)

The degree of lipid peroxidation was assayed in terms of TBA reactive substances. Standard phosphotungstic acid method (10) was used with slight modification (11). In brief, 500 μ l of 10% liver homogenate was taken in a tube containing 0.5ml of 3% SDS, 0.2ml of 0.1 N HCl and 0.3ml of 10% PTA. It was mixed properly and then 1ml TBA (0.67% in 50% acetic acid) was added. It was mixed and heated for 30 minutes in a boiling water bath. The colour was extracted with 4ml butanol and

absorbance was taken at 535 nm. The quantity of TBARS was expressed as nM/100mg protein.

Estimation of Glutathione (GSH)

Reduced GSH was estimated by using standard method of Ellman *et al* (9). 250 μ l of incubation mixture was transferred to a tube containing 0.5 ml of precipitating buffer (5% TCA in 1 mM EDTA). The sample was centrifuged at 2000 g for 10 min and supernatant was collected. It was mixed with 2.5 ml of 0.1 M phosphate buffer (pH 8.0) and colour was developed by adding 0.01% of 100 ml DTNB (5,5 - Dithiobis (2-nitrobenzoic acid). Absorbance was determined at 412 nm using Jasco spectrophotometer as described earlier (12). The GSH values are expressed as mg/g of liver tissue.

Estimation of Superoxide dismutase (SOD)

The activity of SOD was assayed by the reduction of nitro blue tetrazolium (NBT) to blue formazan (13). Here the superoxides were produced by riboflavin mediated photochemical reaction system. In brief, reaction mixture contained 1.7 ml of phosphate buffer (pH 7.8), 150 μ l EDTA (10 mM), 600 μ l methionine (130 mM), 300 μ l NBT (750 mM), and different concentrations of drug. Reaction was initiated by adding 200 μ l riboflavin (60 mM) and placing the tube in front of fluorescent light. A system, devoid of any superoxide-radical scavenger was used as a positive control to compare the results. Absorbance was taken at 560 nm at different time intervals as described earlier (14). The activity of SOD are presented as units/mg protein.

RESULTS

Effect on survival

Different doses of Tamra bhasma were orally given to rats with an objective to determine the LD₅₀. No death was noticed up to 30 days at a dose of 480 mg per 100 g body weight (Table 1).

Effect on liver and kidney functions

Oral administration of Tamra bhasma at a dose of 1000mg/kg body weight for 3 days did not show any change in the activity of serum GOT, GPT, alkaline phosphatase, lactate dehydrogenase, serum urea and serum creatinine. However in similar conditions, 100mg/kg body weight for 15 days showed slight increase in all these enzymes but a dose of 2mg/kg body wt. for 30 days and 5mg/kg body weight even for 90 days, did not show any

change in these enzymes and metabolites. These doses were completely safe. Although there was slight increase in the serum urea in the dose of 100mg for 30 days treatment, but it was statistically insignificant. Interestingly in all the cases the survival was 100% (Table 2).

Effect on histology of liver and kidney

There was no significant change up to the dose of 20mg/kg body weight, when given for 30 days. However on higher doses, necrosis and inflammation was noticed in kidney and liver (Table 3). Results of 2mg/kg body weight for 30 days and 5 mg/kg body weight for 90 days were completely safe and did not show any change in the histological studies.

Effect on different antioxidant parameters

This experiment was carried out for 30 days by using different doses of Tamra bhasma through oral route. There was no increase in the level of lipid peroxidation as liver TBA-RS value was in the normal range, in the dose of 7.5 mg/kg body weight and below, but on higher doses there was dose dependent increase (Table 4). Similarly on higher dose, there was rapid rate of aerial oxidation of GSH in the liver homogenate, but it was significantly maintained in the case of 5mg/kg dose. The initial value remained unchanged even up to 30 days treatment (Table 5). Interestingly the changes in the activity of superoxide dismutase was biphasic that is, on the doses below 5 mg/kg body weight, there was decrease in the activity of SOD, where as on higher doses it gradually increased (Table 6). This increase was also accompanied with that of the degree of lipid peroxidation and the rapid rate of GSH oxidation.

DISCUSSION

It is reported that, Tamra bhasma potentiates the antioxidant activity of animals, when given orally. The treated animals showed less degree of CHP induced lipid peroxidation(5). Interestingly there was no study on higher doses with reference to its toxicity and therapeutic efficacy in regards to its antioxidant property. Worldwide debate is on for the use the ayurvedic metallic preparations. Therefore this study becomes more relevant. Our results clearly indicated that Tamra bhasma does have

antioxidant property in low doses, without any side effect, even up to 90 days of treatment in the dose of 5mg/kg body weight. However in higher doses, when given for a longer period, it induced lipid peroxidation, without any effect on the rate of survival (Table 1) but these tested doses are much higher than the human therapeutic doses. This data is consistent with the literature report, where its use for longer duration at a time is contraindicated(15). The hepatocellular enzymes in serum also showed no significant rise in their activity when given for 90 days in a low dose of 5mg/kg body weight. However 100mg/kg body weight dose for 15 days did show slight adverse effect. Regarding the protection of reduced glutathione, it was found that Tamra bhasma enhances the initial value of GSH in all the animals of drug treated group. In lower doses (5 mg/kg body weight and below) it protects GSH against the aerial oxidation but on higher doses it has no protective response. In the case of superoxide dismutase a biphasic response was observed. In the lower doses (5mg/kg body weight) the SOD activity in the liver homogenate was lower but on higher doses the activity was enhanced. This could be because SOD has a property of substrate induced induction. In lower doses it is not producing any kind of superoxide therefore the SOD activity remains in the normal range but at higher doses, the bhasma might be generating this radical as a side effect, which may be the root cause of enhanced SOD activity. This adverse effect in higher doses is also supported by the enhanced lipid peroxidation. Taking all the observations into consideration, it could be concluded that Tamra bhasma potentiates the antioxidant activity of an animal, when given in lower doses. The myth of toxicity of these preparations in the therapeutic doses should be ruled out because it has not shown any change in the histology of liver and kidney. The SGOT and SGPT, alkaline phosphatase and LDH have also not changed. Further various claims of Tamra bhasma, mentioned in Ayurveda could be explained by its antioxidant property.

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Table 1

Effect of *Tamra bhasma* on the survival of albino rats up to 30 days

S. No.	Dose of bhasma (per kg body weight)	% of survival
1	Drug vector	100%
2	5 mg	100%
3	10 mg	100%
4	20 mg	100%
5	200 mg	100%
6	600 mg	100%
7	1200 mg	100%
8	2400 mg	100%
9	4800 mg	100%

The % has been calculated on the basis of the mean value of six separate experiments. Drug vector is 20 % gum acasia in distilled water

Table 2

Effect of *Tamra bhasma* on liver function and kidney function

Bhasma dose (per Kg body weight)	SGOT (U/ml)	SGPT (U/ml)	SALP (I.U.)	LDH (U/L)	Serum Urea (mg/100ml)	Serum Creatinine (mg/100ml)
Sham Control (Drug vehicle-1ml)	30.52±5.28	17.06±4.85	127.47±0.82	198.17±25.5	35.36±4.67	0.6±0.02
Tamra bhasma 1000mg for 3 days	28.04±3.85	18.35±6.38	124.90±5.77	200.49±21.3	38.74±3.21	0.62±0.12
Tamra bhasma 100mg for 15 days	38.25±6.45*	28.62±4.63*	149.22±5.87*	241.24±2.14*	42.26±6.74	0.52±0.08
Tamra bhasma 2mg for 30 days	29.24±4.63	17.64±5.21	130.24±4.26	195.23±3.47	34.18±4.27	0.61±0.04
Tamra bhasma 5mg for 90 days	30.45±6.32	18.15±3.62	128.95±5.15	199.06±2.17	37.32±5.12	0.57±0.82

Table 3

Histopathological changes of liver and kidney in different doses *Tamra bhasma* on 30 days treated albino rats.

S.No.	Dose of bhasma (mg/kg body weight)	Liver	Kidney
1.	Control (Drug vehicle)	Normal	Normal
2.	5 mg	Normal	Normal
3.	10 mg	Normal	Normal
4.	20 mg	Normal	Normal
5.	200 mg	Normal	Congestion, cloudy swelling 4*
6.	300 mg	Congestion 4*	Congestion within glomeruli & blood vessels, cloudy swelling 5*
7.	600 mg	Focal liver cell necrosis 6*	Cellularity of glomerulie increased, congestion, cloudy swelling 6*

*Number of animals affected out of 6 animals

Table 4

Dose and Time response of *Tamra bhasma* on lipid peroxidation in rat liver homogenate

S.No.	Drug Dose mg/kg BW	7 days treated	15 days treated	30 days treated
1.	Control	593.82 ± 9.19	593.82 ± 9.19	593.82 ± 9.19
2.	2.5 mg	468.82 ± 6.92*	418.64 ± 8.67*	388.12 ± 11.65*
3.	5.0 mg	431.26 ± 6.92*	388.37 ± 9.02*	353.26 ± 7.34*
4.	7.5 mg	456.28 ± 9.31*	492.87 ± 7.16*	503.74 ± 10.64*
3.	10 mg	601.23 ± 8.12	620.49 ± 7.50	613.39 ± 5.56
4.	20 mg	682.75 ± 6.73	675.49 ± 5.32	640.89 ± 6.14
5.	100 mg	751.81 ± 6.14	725.26 ± 7.41	698.72 ± 7.97
6.	200 mg	803.48 ± 5.32	796.84 ± 7.50	785.94 ± 8.93
7.	300 mg	834.77 ± 5.32	815.33 ± 7.34	803.95 ± 7.34
8.	600 mg	1057.56 ± 6.58	1032.91 ± 6.59	1022.96 ± 7.34

The value of TBARS has been represented as nM/100mg protein (Mean ± S.D., n=6)

Table 5

Dose and Time response of Tamara bhasma on glutathione level and its aerial oxidation in rat liver homogenate.

S.No	Dose of bhasma mg/kg b.w.	Oxidation in minutes					
		7 days treated		15 days treated		30 days treated	
		Initial	20	Initial	20	Initial	20
1.	Control	22.29 ± 0.27	10.96 ± 0.27	22.29 ± 0.31	10.96 ± 0.29	22.29 ± 0.25	10.96 ± 0.16
2.	5 mg	27.74 ± 0.23*	16.03 ± 0.19*	21.98 ± 0.18*	18.93 ± 0.17*	23.25 ± 0.19*	17.04 ± 0.21*
3.	10 mg	26.62 ± 0.21	9.65 ± 0.23	22.52 ± 0.21	9.74 ± 0.30	21.22 ± 0.22	8.78 ± 0.25
4.	20 mg	25.92 ± 0.24	8.58 ± 0.22	26.08 ± 0.21	8.03 ± 0.23	25.30 ± 0.13	6.43 ± 0.13
5.	100 mg	24.72 ± 0.17	7.01 ± 0.23	25.03 ± 0.22	7.27 ± 0.22	22.58 ± 0.26	6.14 ± 0.17
6.	200 mg	22.50 ± 0.20	5.06 ± 0.21	24.67 ± 0.35	7.05 ± 0.32	22.51 ± 0.19	5.78 ± 0.17
7.	300 mg	21.59 ± 0.32	4.87 ± 0.21	24.30 ± 0.35	6.05 ± 0.24	22.08 ± 0.18	2.97 ± 0.15
8.	600 mg	20.68 ± 0.35	4.60 ± 0.22	22.12 ± 0.35	4.04 ± 0.22	21.11 ± 0.20	2.29 ± 0.20

Initial value indicates the *in vivo* effect of drug treatment and 20 minutes reading indicates the aerial oxidation of GSH in the petri dish within 20 minutes

The value of reduced glutathione has been represented as mg/gm of liver

(Mean ± S.D. n=6) *p<0.001

Table 6

Dose and Time response of Tamra bhasma on super oxide dismutase activity in rat liver homogenate

S.No	Dose of bhasma mg/kg	SOD activity (units/mg protein)		
		7 days treated	15 days treated	30 days treated
		4min	4min	4min
1.	Control	25.65 ± 0.380	25.65 ± 0.380	25.65 ± 0.380
2.	5mg	18.56 ± 0.349	16.76 ± 0.223	14.59 ± 0.263*
3.	10mg	26.28 ± 0.443	26.31 ± 0.326	26.67 ± 0.250
4.	20mg	28.17 ± 0.323	27.32 ± 0.223	28.06 ± 0.182
5.	100mg	30.23 ± 0.281	29.54 ± 0.343	31.46 ± 0.296
6.	200mg	31.25 ± 0.365	31.13 ± 0.231	32.76 ± 0.239
7.	300mg	32.01 ± 0.258	32.08 ± 0.219	34.90 ± 0.229
8.	600mg	33.03 ± 0.213	34.56 ± 0.308	35.53 ± 0.232

The value of SOD activity has been represented as *Units/mg protein* (Mean ± S.D., n=6), *p<0.001

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