

Embryo protective effect of pomegranate (*Punica granatum* L.) fruit extract in adriamycin-induced oxidative stress

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The possible protective role of pomegranate (*Punica granatum* L.) fruit extract which has shown antioxidant capacity higher than that of red wine and green tea was evaluated against adriamycin-induced oxidative stress in chick embryos. Adriamycin (ADR), an anthracycline broad spectrum of chemotherapeutic drug is used for the treatment of variety of cancers; however, its prolonged use is limited by an irreversible, dose-dependant and progressive cardiomyopathy, hepatotoxicity and general toxicity to other organs in human beings, due to oxidative stress. The morphological changes (malformation of different organs), changes in body weight, volume of amniotic fluid (AF) and biochemical parameters of AF were studied after 24 and 48 h of incubation by comparing ADR alone and pomegranate fruit extract pretreated groups with their respective controls of 12 days old chick embryos. ADR alone at a dose of 70 µg/egg showed a significant dose versus time- dependent reduction in body weight, volume of AF. A dose-related increase in embryo gross morphological deformities and significant changes in the levels of biochemical parameters in AF were observed in ADR-treated group. These changes were significantly ameliorated to normal by pre-administration of pomegranate fruit extract at a dose of 200 µg/egg. Thus, the present study demonstrated the embryo protective nature of pomegranate fruit extract against ADR-induced oxidative stress.

Keywords: Adriamycin, Amniotic fluid, Biochemical parameters, Chick embryo.

Antineoplastic agents may produce adverse side effects during embryonic development in humans and experimental animals. Adriamycin (ADR), one such broad spectrum anticancer drug is one of the most active antineoplastic agent developed to-date for the treatment of soft and solid tumors including acute leukemia's, malignant lymphomas and particularly breast, bladder, endometrial, head and neck, hepatic and prostate cancers¹. The mechanisms underlying the anticancer effect of ADR include redox cycling of the ADR semiquinone radical, DNA intercalation and interference with the function of DNA topoisomerase II and inhibition of RNA and protein synthesis². ADR induces oxidative stress by the generation of reactive oxygen species through redox cycling. Within the cell, it undergoes bioreductive activation and possibly interaction with several oxido-reductases³. The drug toxicity is of great concern during pregnancy period.

ADR is known to be mutagenic, genotoxic and carcinogenic at higher doses. The morphological effects of ADR on chick, mice and rat embryos *in vivo* are well recognized⁴⁻⁶.

A major advantage of using chick embryo as a model in teratology and experimental biology is that one can window the egg, examine the embryo, and precisely target exposure to its specific developmental stages⁷. The amniotic fluid surrounding the embryo has a very complex dynamic nature, functions to equalize the pressure, cushions the fetus from external trauma, participates in fetal biochemical homeostasis and helps in proper growth and mobility of the embryo during its development⁸. Administration of anticancer drugs such as ADR during embryonic stage to animals has been shown to cause fetal malformations. Amniotic fluid studies have wide use in clinical diagnosis and management, and analysis of amniotic fluid has been considered an index of fetal status *in utero*⁹.

The pomegranate (*Punica granatum* L.), commonly known as the "jewel of winter" has recently been acclaimed for its health benefits, in particular for its antioxidant potential. Pomegranate fruit contains higher antioxidants than most other fruit-related food

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Abbreviations: ADR, adriamycin; AF, amniotic fluid; Pg, pomegranate; ED, embryonic development; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ROS, reactive oxygen species.

items¹⁰. The fruit juice has shown chemopreventive, chemotherapeutic, anti-atherosclerotic and anti-inflammatory properties¹¹⁻¹³ and thus fruit consumption has grown tremendously.

In the present investigation, ADR-induced chick embryo mortality, gross malformations and alterations in biochemical parameters of amniotic fluid, due to drug toxicity in developing fetus have been studied. The embryo protective effect of pomegranate fruit is also reported.

Materials and Methods

Chemicals

Adriamycin (ADR) was gifted by Prof. B Nagarajan, Head, Department of Tumor Biochemistry and Microbiology, Cancer Institute, Adyar, Chennai, India. All other chemicals were of analytical grade and obtained from Qualigens and Merck, Mumbai, India.

Maintenance of eggs

Freshly laid zero-day old fertilized eggs of Bobcock strain were procured from Govt. Veterinary University, Tirupati, and also from Balaji hatcheries, Chittoor District, Andhra Pradesh, India. The eggs were cleaned with distilled water and alcohol and set in an egg incubator with the temperature at 38°C and the relative humidity at 58-60%. The day, the eggs were set was designated as E0. The humidity of incubator was maintained by keeping the tray full of water inside. The eggs were rotated manually and examined through the candler every day for the proper growth and viability. The dead embryos in the eggs were removed immediately from the incubator to ensure proper growth of remaining embryos in the eggs. To locate an injection site for drug administration, the eggs were candled and a site that avoided membrane-bound blood vessels was marked about 2.0 cm below the air cell¹⁴.

Embryo toxicity study

The 12 days old chick embryos taken for the study were divided into 4 groups containing six animals each. Based on LD₅₀ values, embryos were administered with ADR (50, 60 and 70 µg) on 12th day and normal saline for control embryos by injecting into the air sac and designated as ED-12. After ADR administration, the injection site was sealed with paraffin and the eggs were returned to the incubator for further incubation. The morphological changes (malformation of different organs), change in

volume of amniotic fluid and body weight were studied at 24 and 48 h of incubation by comparing ADR-treated groups with their respective control.

Preparation of pomegranate fruit extract

Fresh pomegranate fruits (Telugu name: *Dhanimma*) were purchased from a local market at Tirupati and their identity was authenticated in the Department of Botany, Sri Venkateswara University, Tirupati, Chittoor District, Andhra Pradesh. The fruits, excluding the peel were macerated in 50% (v/v) ethanol solution in a ratio of 1:3 (w/v) and kept for 15 days. The extract was filtered and ethanol was removed by vacuum evaporation on a rotary evaporator at 55°C. The aqueous extract was then lyophilized and stored at -20°C until used. The yield of extract was 10%¹⁵.

Experimental design

In the present study, chick embryos were divided into 5 groups of six animals each. Group I received normal saline (served as controls), Group II received only hydroalcoholic extract of fruit at a dose of 200 µg/egg, Group III received only ADR at a dose of 70 µg/egg, and Groups IV and V received pre administration of hydroalcoholic extract of pomegranate at a dose of 100 and 200 µg 6 h prior to the ADR 70 µg treatment. The protective role of fruit extract was assessed after 24 and 48 h of incubation.

Collection of embryonic tissues and amniotic fluid

After the experimental period, 12 days old embryos were sacrificed by opening at air sac. Amniotic fluid and tissues were collected aseptically, stored in chilled cold saline at -20°C and used for biochemical analysis.

Biochemical analysis

Amniotic fluid was collected from the incubated eggs and centrifuged at 3000 rpm for 10 min to remove cell debris. Clear supernatant was taken for the biochemical assays. Protein content¹⁶, phosphorous content¹⁷, glucose¹⁸, sodium and potassium¹⁹, calcium¹⁹, urea²⁰, uric acid²¹ and creatinine²² were assayed in amniotic fluid. The activities of marker enzymes, such as alkaline phosphatase (ALP)²³, alanine aminotransferase (ALT)²⁴ and aspartate aminotransferase (AST)²⁴ were also estimated in amniotic fluid.

Statistical analysis

All the results were expressed as mean ± standard error (SE) for six animals in each group and the

difference between groups were considered significant, when the P value determined student's 't' value was less than 0.05 and 0.01.

Results and Discussion

Adriamycin treatment on 12 days old embryos showed a significant dose versus time dependent reduction in volume of amniotic fluid as well as body weight (Table 1) of chick embryos when compared with control. This clearly indicates the embryo toxicity generated by the drug during developmental changes.

In present study, a single injection of different doses (50, 60 and 70 μg) of ADR was made directly into the air sac. The significant decrease in weight of ADR-treated group was due to the decreased energy supply as evidenced by the altered properties of macromolecules of yolk-sac are extraembryonic vascular network which, in turn, reduce the nutrient transfer and hence responsible for affecting the embryonic growth when compared to controls. There is an antioxidant/prooxidant balance in tissues during chick embryo development, which is responsible for normal embryonic development and post-hatch chick

viability²⁵. ADR induces free radical generation, which along with reactive metabolites are either detoxified through conjugating with glutathione, which is considered to be critical in protection against embryotoxicants²⁶, otherwise reacts with tissue macromolecules and initiates its damage. Toxic effects of ADR on chick embryos at higher doses (70 μg) and less effect at lower doses (50 μg) indicate that ADR is metabolized to toxic metabolites, which at lower doses can be detoxified by conjugating with glutathione but at higher doses are unable to detoxify as the glutathione level in tissues depletes throughout the embryonic development. Alterations in AF volume due to various teratogenic agents have been reported²⁷. A significant decrease in the volume of amniotic fluid is believed to be caused by less albumin entering the amniotic fluid via sero-amniotic connection²⁸. The conditions that retard the perforation of sero-amniotic plate also affect the fluctuations in AF volume.

Morphological deformities observed

12 days old chick embryos were examined for any malformations and mortality rate effects caused by

Table 1—Weight and volume of 12 days old chick embryo at 24 and 48 h of ADR treatment

[Values mean \pm SE and are average of six sets of separate experiments]

	Day of exposure (12 th day)	Group I (Control)	Group II (ADR 50 μg)	Group III (ADR 60 μg)	Group IV (ADR 70 μg)
Weight of embryo (mg/egg wt)	After 24 h	7.11 \pm 0.06	5.97 \pm 0.15*	5.17 \pm 0.14**	4.08 \pm 0.14**
	After 48 h	9.40 \pm 0.10	7.83 \pm 0.09**	5.91 \pm 0.20**	3.36 \pm 0.11**
Volume of amniotic fluid (ml/embryo)	After 24 h	4.43 \pm 0.09	3.68 \pm 0.05*	2.75 \pm 0.05**	2.25 \pm 0.04**
	After 48 h	4.00 \pm 0.08	3.06 \pm 0.06*	2.66 \pm 0.05**	1.96 \pm 0.04**

Statistically significant alterations are expressed as * $P < 0.05$, ** $P < 0.01$, NS, not significant

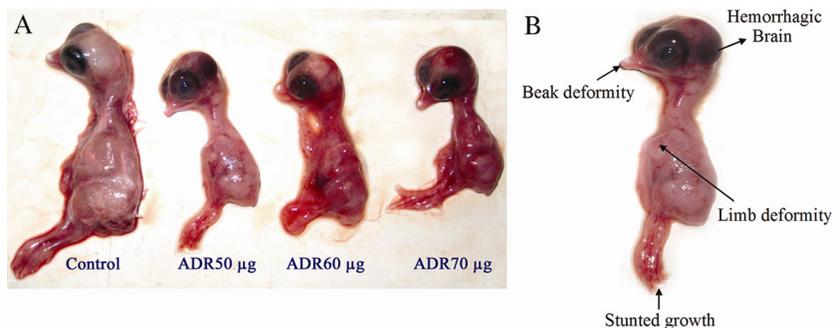


Fig. 1—(A): Effect of ADR on 12 day old chick embryo at 48 hours treatment [The embryos treated on 12 day showed weight loss, limb deformities, hydrocephalous, hemorrhagic brain and short wings]; and (B): Effect of 70 μg ADR on 12 day old chick embryo at 48 h of treatment.

ADR after 24 and 48 h of treatment. No mortality and deformities were observed in the control group, whereas in ADR-treated group, the spectrum of malformations in the embryos such as scanty feathers, limb deformities, short wings, beak deformities, hemorrhagic brain and fluids, hydrocephalous, thickening of neck and immediate death were observed. Stunted growth was the major malformation observed with teratogenic dose (ADR 70 µg) (Fig 1A and B).

In present study, chick embryos were selected since not much study was being carried out by administering ADR. Administration of different doses of ADR (50, 60 and 70 µg) to 12 days old embryo caused a dose-dependent increase in embryo mortality, gross morphological damage and reduction in fetal weight. ADR exerts a dose-response effect on the different regions of the embryo. The sensitivity of different organs varies according to the dose of ADR

used and sometimes shown susceptibility at a lower dose. The embryo toxicity of ADR *in vitro*, by exposing cultured rat embryos at low concentrations of ADR has shown a decrease in growth parameters, such as somite numbers, embryonic length²⁹. As far as chick embryonic model concerned, many studies have been carried out in the recent years by administering other antineoplastic agents such as chlorambucil and cytarbine also noticed malformations induced in chick embryos³⁰⁻³². The malformations in chick embryos correlate with the result of an earlier study³³. Growth retardation and internal hemorrhage represent the most frequent malformation. Growing embryo needs a lot of energy as the cells multiply rapidly.

Effect of pretreatment of pomegranate fruit extract on ADR-induced biochemical changes in chick embryos at 24 and 48 h of treatment

Administration of ADR caused significant biochemical changes in AF (Table 2). No significant

Table 2—Protective effect of pomegranate extract on biochemical parameters in 12 days-old chick embryonic amniotic fluid at 24 h and 48 h adriamycin treatment [Values are average of six sets of separate experiments, mean ± SE].

	Glucose (mg/dL)	Protein (mg/dL)	Uric acid (mg/dL)	Urea (mg/dL)	Creatinine (mg/dL)	Sodium (mEq/L)	Potassium (mEq/L)	Inorganic phosphorus (mg/dL)	Calcium (mg/dL)	Cholesterol (mg/dL)	ALP (IU/L)	ALT (IU/L)	AST (IU/L)
After 24 h ADR treatment													
Group-I (Control)	22.92 ±0.11	30.15 ±0.10	12.02 ±0.20	5.05 ±0.07	0.83 ±0.06	107.98 ±0.10	3.37 ±0.08	0.42 ±0.04	3.65 ±0.08	20.87 ±0.11	5.82 ±0.05	16.72 ±0.08	5.42 ±0.07
Group-II (Pg 200 mg)	23.02 ±0.11	30.70 ±0.09	12.75 ±0.11	5.42 ±0.16	1.02 ±0.05	107.30 ±0.21	3.58 ±0.09	0.50 ±0.06	3.42 ±0.05	20.63 ±0.07	6.00 ±0.09	17.03 ±0.11	5.85 ±0.10
Group-III (ADR 70 mg)	33.37 ±0.17	38.08 ±0.07	19.25 ±0.07	12.40 ±0.09	2.32 ±0.05	101.27 ±0.26	6.53 ±0.08	1.27 ±0.04	1.90 ±0.08	15.00 ±0.06	13.15 ±0.09	28.67 ±0.08	11.28 ±0.09
Group-IV (Pg 100 mg 6 h prior to ADR 70 mg)	30.03 ±0.13**	37.12 ±0.05**	18.35 ±0.016*	11.33 ±0.10**	2.17 ±0.05NS	102.78 ±0.04*	5.70 ±0.05*	0.98 ±0.05*	2.22 ±0.08*	16.18 ±0.06**	11.13 ±0.06**	26.51 ±0.10**	10.60 ±0.05**
Group-V (Pg 200 mg 6 h prior to ADR 70 mg)	23.98 ±0.64**	31.31 ±0.10**	12.68 ±0.09**	5.88 ±0.13**	0.96 ±0.07**	107.00 ±0.18**	3.70 ±0.06**	0.56 ±0.06**	3.26 ±0.06**	19.68 ±0.08**	6.21 ±0.06**	17.46 ±0.09**	6.01 ±0.05**
After 48 h ADR treatment													
Group-I (Control)	24.53 ±0.11	43.28 ±0.17	15.47 ±0.13	6.85 ±0.12	1.23 ±0.09	121.83 ±0.11	4.43 ±0.12	1.08 ±0.13	5.98 ±0.09	26.88 ±0.12	7.38 ±0.14	20.32 ±0.12	12.13 ±0.15
Group-II (Pg- 200 mg)	24.92 ±0.15	43.88 ±0.13	15.88 ±0.08	7.23 ±0.08	1.38 ±0.06	121.38 ±0.09	5.05 ±0.07	1.52 ±0.05	5.43 ±0.07	26.43 ±0.13	8.00 ±0.09	21.00 ±0.08	12.53 ±0.19
Group-III (ADR 70 mg)	34.12 ±0.08	65.25 ±0.10	25.07 ±0.14	15.77 ±0.15	3.33 ±0.10	83.83 ±1.32	12.13 ±0.19	3.22 ±0.08	2.38 ±0.10	20.75 ±0.08	14.57 ±0.06	34.43 ±0.14	29.43 ±0.10
Group-IV (Pg 100 mg 6 h prior to ADR 70 mg)	32.31 ±0.12**	62.08 ±0.22**	23.82 ±0.16**	13.57 ±0.10**	2.43 ±0.11*	96.00 ±0.66**	10.43 ±0.11**	2.38 ±0.09*	3.21 ±0.11*	22.15 ±0.14**	13.43 ±0.11*	30.23 ±0.16**	26.30 ±0.10**
Group-V (Pg 200 mg 6 h prior to ADR 70 mg)	25.48 ±0.13**	44.00 ±0.09**	16.00 ±0.14**	7.48 ±0.12**	1.41 ±0.09**	121.00 ±0.09**	5.21 ±0.05**	1.53 ±0.06**	5.05 ±0.13**	26.31 ±0.07**	8.30 ±0.06**	21.58 ±0.16**	13.01 ±0.08**

Group I embryos received normal saline (served as controls), Group II only hydroalcoholic extract of pomegranate at a dose of 200 µg/egg, Group III ADR at a dose of 70 mg/egg, Group IV and V received pre administration of hydroalcoholic extract of pomegranate at a dose of 100 µg and 200 µg 6 h prior to the ADR 70 mg treatment. Statistically significant alterations are expressed as * $P < 0.05$; ** $P < 0.01$; NS, Not-significant. For statistical evaluation of significant variations, comparisons were made between Group IV and V against Group III. There was no significant difference between Group II and I.

alterations of biochemical parameters were observed in group II animals, when compared to group I animals. In ADR-treated group III, significant elevation ($P<0.01$) in levels of cellular and non-protein nitrogen components such as urea, uric acid and creatinine as well as in protein, potassium and inorganic phosphorus levels was observed. Elevated levels of urea, uric acid and creatinine in ADR-treated group might be due to the damage caused by ADR on the end product of nitrogen metabolism. Uric acid, the metabolic end product of purine metabolism is a selective antioxidant, capable of reacting with free radicals and hypochlorous acid³⁴. An elevated level of creatinine is an evidence of marked impairment of kidney function and its retention is thus an index of glomerular insufficiency³⁵. All these levels were significantly ($P<0.01$) normalized, when pretreated with pomegranate extract (groups IV and V) in a dose-dependent manner at 24 and 48 h of incubation.

A significant decrease ($P<0.01$) in the levels of sodium, calcium and cholesterol was observed in ADR-treated group III. Pre-administration of pomegranate extract significantly increased ($P<0.05$, 0.01) the levels in group IV and V, when compared with group III animals. The glucose levels in AF were significantly increased in ADR-treated animals, compared to controls in a dose-dependent manner. The change in the level of glucose is an indication of alterations in glucose metabolism and the increased levels in AF might be due to changes in membrane permeability and diffusion of embryonic glucose into AF^{31,32}. The elevated levels of proteins in AF indicated the leakage of RBC cells into AF. These levels were reversed to almost near normal by pretreatment with pomegranate extracts.

A significant increase ($P<0.01$) in the ALP, ALT and AST activities was observed in ADR-treated animals. Increased levels of marker enzymes of AF on ADR administration might be due to the effect of ADR on normal hepatocytes and cardiac cells which cause possible leakage of these enzymes to AF. ALP is the prototype of hepatic marker enzyme that reflects the pathological alterations in bile flow³⁶. ALP, ALT and AST levels were significantly reduced ($P<0.01$) in a dose-dependent manner, when pretreated with pomegranate extracts. In a similar study¹⁵, no significant change in AST and ALT levels was observed during administration of pomegranate hydroalcoholic extract to the rats.

The fluctuations in the Na/K ratio of AF might reflect that of the blood and due to the effect of yolk concentration during embryonic development³⁷. These levels were reversed to the near normal with pretreatment of pomegranate fruit extract. The decreased level of calcium and cholesterol in ADR-treated animals explains the effect of ADR on AF, involved in skeletal development and embryonic growth during the gestation period. The reversal of calcium and cholesterol levels was observed with pomegranate extract administration.

Conclusion

The present study demonstrated that pre-administration of pomegranate fruit extract (200 $\mu\text{g}/\text{egg}$) showed beneficial effects in minimizing the malformations by maintaining prooxidant/antioxidant balance and restoring biochemical variables against ADR-induced oxidative stress in chick embryos.

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