



Attenuating Effect of Vitamin E and *Tinospora cordifolia* on Bisphenol-A Induced Apoptosis in Goat Testis

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ABSTRACT

During the present study effect of Vitamin E and *Tinospora cordifolia* on BPA induced goat (*Capra hircus*) testicular tissue was evaluated. Testicular tissue was exposed to varied concentrations of BPA, Vitamin E and *Tinospora cordifolia* in three experimental groups along with their specific control for 4 and 8hrs. In group I, the cultured tissue was exposed to three doses of Bisphenol-A viz. 0.01, 1.0 and 100 nM/ml. In group II, Vitamin E (0.1 μM/ml) was supplemented along with BPA (0.01, 1.0, 100 nM/ml) and in group III *Tinospora cordifolia* extract (250 μg/ml) was added with BPA (0.01, 1.0, 100 nM/ml) in the culture media. With the increase in dose level as well as the exposure duration, the frequency of apoptotic cells increased significantly ($p < 0.05$), as revealed by acridine orange and methylene blue staining. BPA exposure also induced a decline in the antioxidant enzyme activity such as catalase, glutathione peroxidase and reduced glutathione. Vitamin E and *Tinospora cordifolia* resulted in a decrease in the percentage of apoptotic cells and an increase in levels of catalase, glutathione peroxidase and reduced glutathione activity was observed. Hence, it is concluded that BPA induced more damage at its higher concentration and the antioxidant Vitamin E and *Tinospora cordifolia* ameliorate the oxidative stress-induced changes, thereby suggesting the potential of Vitamin E and *Tinospora cordifolia* in counteracting the cellular damage induced by agents like BPA.



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INTRODUCTION

Endocrine Disrupting Chemicals encompass a variety of chemical classes, including pesticides, compounds used in the plastic industry and in consumer products, other industrial by-products and

pollutants. Bisphenol-A is among the most prominent environmental estrogens used worldwide. It is a component employed in the manufacture of two types of polymers used in food contact articles, specifically polycarbonate polymers, epoxy-based enamels and coatings. BPA has been reported to cause reproductive as well as endocrine disruptions in humans (Erlar and Novak, 2010). BPA has been documented as a testicular toxicant that may account for the enhancing frequency of infertility (Takahashi and Oishi, 2001; Tohei et al., 2001).

An array of antioxidant systems viz. catalase, glutathione transferase, peroxidase, reductase, superoxide dismutase defend oxidative stress in testis. A wide variety of EDC's such as BPA, are known to perturb these defenses by generating reactive oxygen species, such as hydroxyl radicals, peroxide anions, peroxy radicals, and hydrogen peroxide (Aitken and Roman, 2008). Hassan et al. (2012) have reported

that high levels of BPA exposure results in increased generation of reactive oxygen species culminating in oxidative stress. El-Beshbishy *et al.* (2013) have also observed that administration of BPA to male rats triggered a decrease in testicular antioxidant enzymes such as glutathione peroxidase, catalase, superoxide dismutase, glutathione reductase and an increase in the level of ROS. An increased formation of ROS in testis may cause significant alterations in tissue physiology or induce oxidative damage to DNA, which is of the potential risk to sperm production and offspring (Gold *et al.*, 1995). Thus, ROS are associated with oxidative stress and are likely to play a significant role in reproductive disorders. Furthermore, reports have documented that BPA exposure was associated with germ cell apoptosis (Takahashi and Oishi, 2003).

BPA significantly disturb the prooxidant-antioxidant balance, therefore, reinforcing ROS scavenging activity in testis by exogenous antioxidants such as Vitamin E is necessary to mitigate the ill effect of BPA. Vitamin E is a lipophilic antioxidant, well recognized for its effective inhibition of lipid peroxidation in foods and living cells (Burton and Traber, 1990). Vitamin E is synthesized only by plants: therefore, it is a very important dietary nutrient for humans and animals (Fryer, 1992). Fang *et al.* (2013) reported that co-administration of BPA with Vitamin E in male mice showed an increase in antioxidant response, which protected against oxidative damage caused by BPA.

Tinospora cordifolia, commonly named "Giloy", is known for its immense application in the treatment of various diseases in the traditional ayurvedic literature. *Tinospora cordifolia* extract has been reported for its strong free radical scavenging properties against superoxide anion (O_2^-), hydroxyl radicals (OH), NO radical, and peroxy nitrite anion (ONOO-) (Rawal *et al.*, 2004). Oral administration of aqueous extract of *Tinospora cordifolia* resulted in a significant reduction in thiobarbituric acid reactive substances (TBARS) and an increase in reduced glutathione (GSH) catalase (CAT) and superoxide dismutase (SOD) in alloxan diabetic rats (Prince and Menon, 2001). Jayaganthan *et al.* (2013) have reported that administration of *Tinospora cordifolia* increased the function of testes, presumably through enhanced secretion of antioxidant enzymes in rams.

It becomes pronounced from the literature that prevalent studies had been accomplished on mice and rats in-vivo whereas, in ruminants like goat are still lagging. What impact these xenobiotics have on the reproductive potential of small ruminants needs

to be analyzed. Keeping in view these lacunae, the present study was conducted to determine the effect of nanomolar concentration of BPA on testicular tissue *in-vitro* and ameliorative potency of Vitamin E and *Tinospora cordifolia*.

MATERIALS AND METHODS

The testes from the mature goat (*Capra hircus*) obtained from slaughterhouses around Kurukshetra (29° 6'N, 76 °50'E) and were brought to the lab at 4°C in normal saline.

Testicular tissue culture

The testis was cut into smaller pieces, approximately 1mm³ in size. The tissue medium constituted TCM-199 and antibiotics (200 unit penicillin 100 IU/ml and streptomycin 100 g/ml).

The experimental set-up had four groups, as shown in Table 1. The culture plates as per experimental setup were incubated at 37°C, 95% humidity in a CO₂ incubator.

Preparation of Testicular Cell Suspension

The testicular tissue culture was harvested after 4 and 8 hours, and tissue was minced with the help of a blade in phosphate buffer saline at pH 7.0. Washing was done thrice with phosphate buffer saline in ultracentrifuge for 5 minutes. The supernatant was discarded. The pellet was mixed with PBS and the cell suspension was further used for apoptotic and biochemical analysis.

APOPTOTIC ASSAY

The apoptotic assay was made to study the change in apoptotic frequency after treatment with different doses of BPA in both the control and experimental groups exposed for 4 and 8 hrs of time duration. For analyzing apoptotic changes acridine orange and methylene blue staining were used.

Acridine Orange Staining

Acridine orange staining was done following the method of Kalia and Bansal (2009). About 10μL of cultured cell suspension was transformed onto a micro slide and stained with a droplet (50μl) of acridine orange. The slides were analyzed under a fluorescence microscope, and selected portion revealing life and dead cells were counted and photographed.

Methylene Blue Staining

Methylene blue staining was performed using Kwolek-Mirek and Zadrag-Tecza (2014) method. After preparation of cell suspension from cultured testicular tissue, cells were transferred onto a micro slide and stained with methylene blue

stain. The slides were observed under a light microscope and portion showing viable and non-viable cells were counted and photographed.

BIOCHEMICAL ANALYSIS

For biochemical analyses, three antioxidant enzymes were studied viz. catalase, glutathione peroxidase, reduced glutathione by the following methods:

Antioxidants Enzymes

Catalase

For catalase estimation, 0.5ml of testicular cell suspension (enzyme source) was incubated with 50 mM of phosphate buffer saline for 30 min at 4°C. The absorbance was recorded at 240nm instantly after the addition of freshly prepared 6 mM H₂O₂ using an IMPLEN nano spectrometer for 3 minutes at an interval of 20 seconds. The decrease in absorbance of H₂O₂ per unit time was directly proportional to the measure of catalase activity (Aebi, 1984). The protein was estimated by Lowry *et al.* (1951).

Glutathione Peroxidase

The reaction mixture consisting of 0.2 ml EDTA, 0.1 ml sodium azide, 0.1 ml of H₂O₂, 0.2 ml of GSH, 0.4 ml phosphate buffer (pH 7.0) and 0.2 ml of enzyme source and 0.8 ml distilled water was incubated at 37°C for 10 minutes. The reaction was inhibited by the addition of 10% TCA and the tubes were centrifuged. To the supernatant, 3.0 ml of 0.3M disodium hydrogen phosphate and 1.0 ml of DTNB were added. The colour developed was read at 420 nm immediately (Rotruck *et al.*, 1973).

GSH assay

0.1 ml of enzyme source was mixed with TCA and kept on ice for few minutes. This mixture was then subjected to centrifugation at 3000 g for 5 minutes. To the supernatant, 0.2 M sodium phosphate buffer (pH 8) and 2 ml of 0.6 mM DTNB (prepared in 0.2 M buffer, pH 8) was added. The yellow colour obtained was recorded at 412 nm against the blank containing TCA instead of supernatant (Moron *et al.*, 1979).

The data were expressed as Mean \pm Standard Error of Mean (S.E.M) with all experiments carried out in triplicate using SPSS 16 statistical software. One way ANOVA with Duncan Post hoc test was used to analyze the significance of differences between the experimental and control observations. Statistical significance was implied at $p < 0.05$.

RESULTS

The changes in the frequency of apoptotic cells and antioxidant enzymes activity were studied in BPA exposed testicular tissue of goat *in vitro*. Acridine orange staining revealed testicular cells with green fluorescence denoting live cells (control), while the cells exhibiting red fluorescence were apoptotic (Figure 1). Apoptotic index analysis of testicular cells showed an increase in the frequency of apoptotic cells with an increase in exposure as well time duration (Table 2). After Vitamin E and *Tinospora cordifolia* supplementation, a decrease in the frequency of apoptotic cells (approximately by 5-10%) was recorded. A similar trend was observed with methylene blue staining, as shown in Figure 2 and Table 3. The testicular tissue treated with different concentrations (0.01, 1nM, 100 nM/ml) of BPA reported a significant decrease ($p < 0.05$) in the activity of glutathione peroxidase as compared to control in the dose and time-dependent manner. After an 8-hour exposure duration, the glutathione peroxidase activity at 100 nM/ml of BPA was found to be minimum, i.e. 8.1 nmol/mg protein, as compared to control, which was 18.5 nmol/mg protein. On supplementation of Vitamin E and *Tinospora cordifolia* with 100 nM/ml dose of BPA, the activity of glutathione peroxidase was significantly ($p < 0.05$) increased to 17.9 and 11.2 nmol/mg protein, respectively (Figure 3). A similar declining pattern was recorded in the catalase and reduced glutathione enzyme assays (Figure 4, Figure 5).

DISCUSSION

During the present study, apoptotic as well as biochemical changes were observed in the testicular cells of the goat, which were exposed to a nanomolar concentration of BPA alone and along with supplementation of Vitamin E and *Tinospora cordifolia* for 4 and 8hr *in vitro*. Apoptotic analysis by acridine orange staining revealed that BPA induces maximum apoptotic damage at the highest concentration, i.e. 100 nM/ml (70.16), followed by 1 nM/ml (62.25) and 0.01 nM/ml (60.06) in testicular cells of goat. There was a significant increase in the frequency of apoptotic cells which was approximately 2-10% ($p < 0.05$). The present findings strongly support the findings of Kalia and Bansal (2008), who have reported that the apoptotic cells increase with a higher dose of diethyl maleate in the testis of mice. The fluorescent staining demarcated that apoptotic cells emitted (red fluorescence) while live cells exhibited (green fluorescence). These results are also in accordance with (Wang *et al.*, 2015), who have also studied that the normal cells with intact

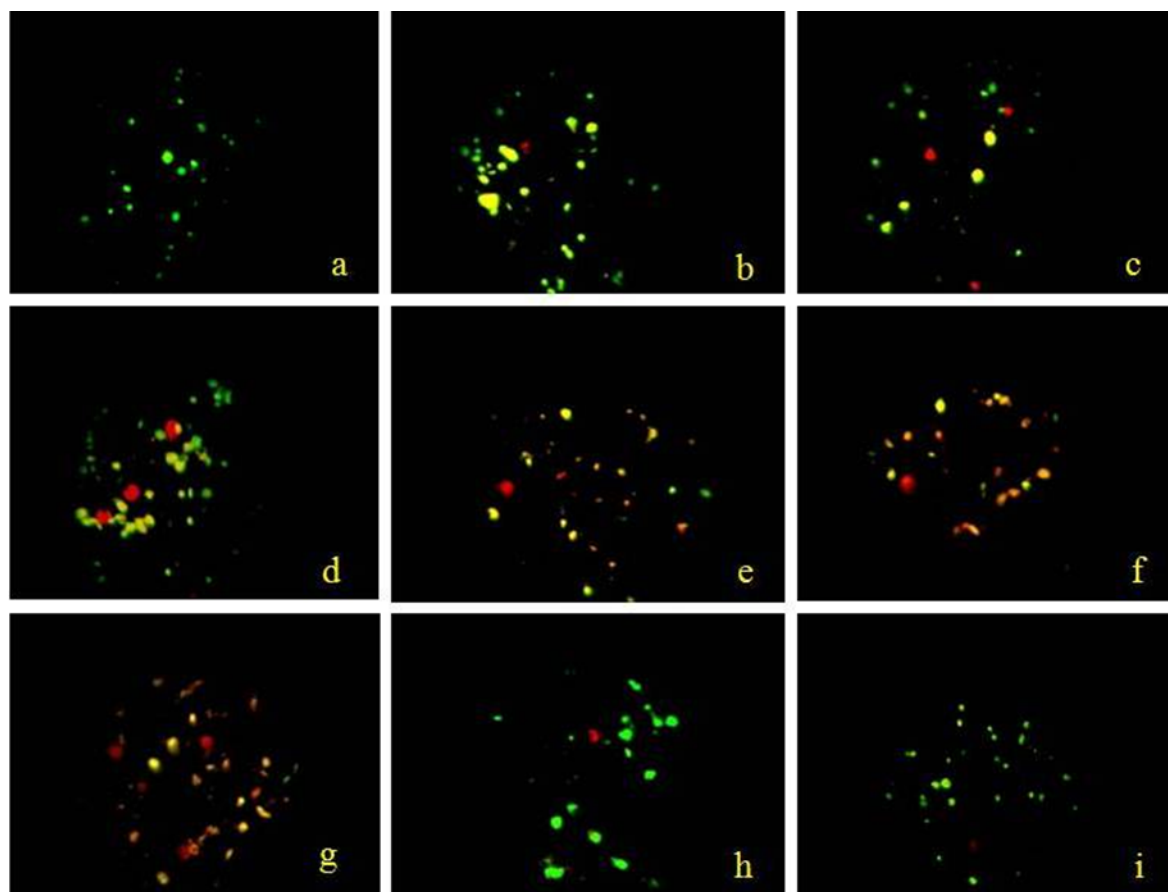


Figure 1: Photomicrographs of testicular cells revealing a) control-live cells (green fluorescence). In (e, f, g) frequency of apoptotic cells (red fluorescence) increased with an increase in dose from 0.01, 1, 100 nM/ml at 8 hr duration as compared to 4 hr (b, c, d). Upon supplementation of Vitamin E and *Tinospora cordifolia* along with BPA, the frequency of apoptotic cells had decreased (h, i)

Table 1: The experimental setup of testicular cell culture *in-vitro*

Culture duration	Group 1	Group 2	Group 3	Group 4
4 and 8hrs.	Culture media + BPA	Culture media + BPA	Culture media + BPA	Culture media only
	0.01 nM/ml	0.01 nM/ml + Vit. E	0.01 nM/ml + T.C.E.	
	1 nM/ml	1 nM/ml + Vit. E	1 nM/ml + T.C.E.	
	100 nM/ml	100 nM/ml + Vit. E	100 nM/ml + T.C.E.	

cell membrane were stained green by AO, whereas the late apoptotic cells were stained red. A similar trend was recorded with methylene blue-stained cells, where dead cells appeared as blue and live were hyaline in color. These observations are in coherence with the findings of [Boyd et al. \(2003\)](#), who have also documented that blue cells were apoptotic while the live cells were white in color. It was observed that BPA induced apoptosis in testicular cells, which led to a significant decline in the level of antioxidant enzymes (glutathione peroxidase, catalase, reduced glutathione) in a time and dose-dependent manner. These results are in agree-

ment with the observations of [Kabuto et al. \(2003\)](#), who have reported that declined level of antioxidant enzymes in male mice upon administration of BPA *in-vivo*.

In the present study, amelioration by Vitamin E showed a significant decrease in the number of apoptotic cells and an increased level of antioxidant enzymes. Hence, exhibited significant recovery in the testicular cells. The results of the present study are in accordance with ([Sharma and Gulati, 2013](#); [Gandhi and Sharma, 2017](#)), who have revealed that administration of Vitamin C and Vitamin E elevated

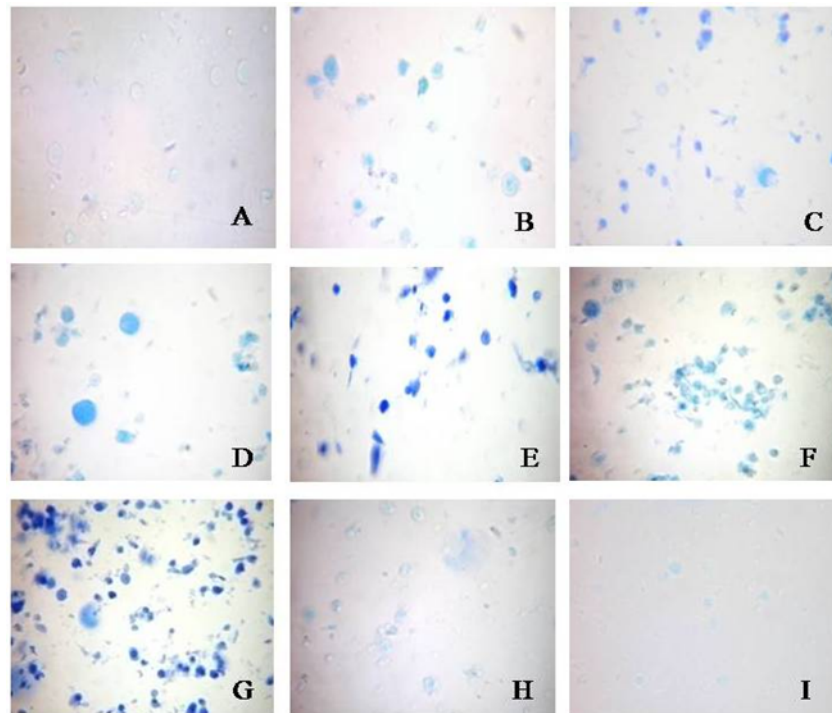


Figure 2: Photomicrographs of testicular cells revealing A) viable cells (colorless). In (E, F, G) frequency of non-viable cells (blue) increased with an increase in dose from 0.01, 1, 100nM/ml at 8 hours culture duration as compared to 4 hrs (B, C, D). Administration of BPA with Vitamin E and *Tinospora cordifolia* showed a decrease in the frequency of apoptotic cells (H, I)

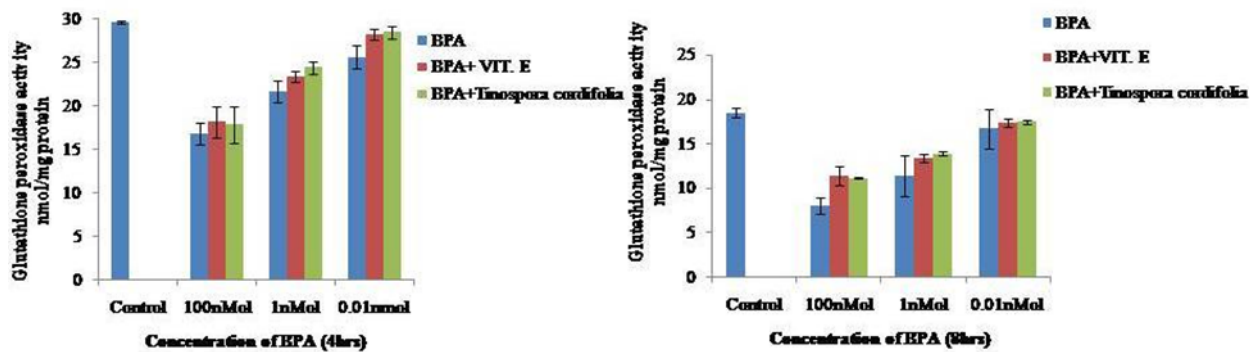


Figure 3: Pattern of changes in the activity of glutathione peroxidase (nmol/mg protein) when exposed to different concentration of BPA and BPA along with Vitamin E and *Tinospira cordifolia* at 4, 8 hrs

the level of Catalase and Superoxide dismutase enzyme activities and thus attenuated malathion induced testicular damage. Therefore, it becomes obvious that BPA manifest its effect in a manner similar to that of malathion. Fang *et al.* (2013) have also observed that the level of antioxidant enzymes viz. GPx, GSH, Catalase had got alleviated upon supplementation of Vitamin E along with BPA and hence it may have a certain protective effect on reproductive inhibition.

The results of the present study have revealed that the goat testicular tissue upon administration

with *Tinospora cordifolia* resulted in a significant increase ($p < 0.05$) in reduced glutathione (GSH), catalase (CAT) and glutathione peroxidase (GPx) levels and showed a decline in a number of apoptotic cells in comparison to the treated groups when stained with acridine orange and methylene blue respectively. The results of the present findings are in agreement with Sangeetha *et al.* (2013), who have reported that oral treatment of *Tinospora Cordifolia* suppressed the oxidative stress marker, thiobarbituric acid reactive substances (TBARS) formation and restored cellular defense anti-oxidant markers,

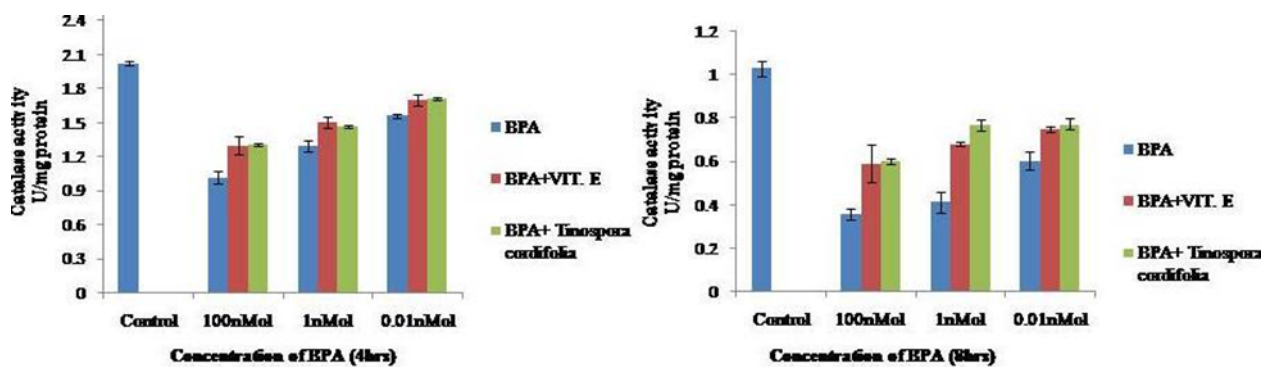


Figure 4: Bar diagram showing the activity of Catalase (U/mg protein) in testicular cells of the goat when exposed to 0.01 to 100 nM/ml dose of BPA and BPA (0.01 to 100 nM/ml) along with Vitamin E and *Tinospora cordifolia* at 4, 8 hrs

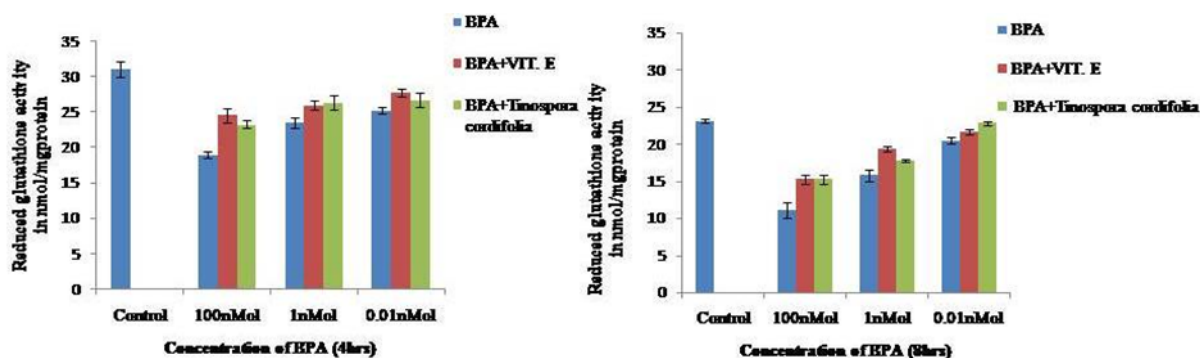


Figure 5: Changes in the activity of reduced glutathione (nmol/mg protein) with different doses of BPA and BPA along with supplementation of Vitamin E and *Tinospora cordifolia* in testicular cells of goat

Table 2: Apoptotic index of testicular cells after treatment with different doses of BPA (0.01 to 100 nM/ml), with supplementation of Vitamin E and *Tinospora cordifolia* by acridine orange at 4, 8 hours culture duration

Time duration	Contro	100 nM/ml			1 nM/ml			0.01 nM/ml		
		BPA	BPA+ VIT. E	BPA+ T.C.E	BPA	BPA+ VIT. E	BPA+ T.C.E.	BPA	BPA+ VIT. E	BPA+ T.C.E
4hrs	22.9	61.50	56.54	53.47	51.49	47.89	46.56	39.51	33.83	33.63
	± 3.75	± 0.64	± 1.00	± 1.20	± 0.75	± 1.19	± 1.42	± 1.21	± 0.89	± 0.72
8hrs	37.43	70.16	62.25	60.06	58.11	52.56	52.36	50.72	44.45	43.38
	± 1.29	± 2.27	± 0.46	± 2.86	± 1.76	± 0.28	± 1.94	± 0.62	± 2.31	± 2.41

Values are expressed as mean ± S.E.M. The values differ significantly (p<0.05) in different concentrations

Table 3: Apoptotic index of methylene blue-stained testicular cells with different doses of BPA (0.01 to 100 nM/ml) and BPA along with supplementation of Vitamin E and *Tinospora cordifolia* by at 4, 8 hrs of culture duration

Time duration	Control	100 nM/ml			1 nM/ml			0.01 nM/ml		
		BPA	BPA+ VIT. E	BPA+ T.C.E	BPA	BPA+ VIT. E	BPA+ T.C.E.	BPA	BPA+ VIT. E	BPA+ T.C.E
4hrs	24.36	60.42	51.05	53.13	50.45	38.97	42.11	42.58	32.10	32.04
	± 1.82	± 1.72	± 1.72	± 1.91	± 1.68	± 3.82	± 1.88	± 1.95	± 2.37	± 1.68
8hrs	38.56	75.23	66.85	61.75	63.37	54.22	50.41	53.56	45.57	47.73
	± 0.76	± 1.80	± 2.12	± 2.27	± 2.12	± 2.32	± 2.16	± 2.67	± 2.08	± 0.45

Values are expressed as mean ± S.E.M. The values differ significantly ($p < 0.05$) in different concentrations

including superoxide dismutase (SOD), glutathione peroxidase (GPx) and reduced glutathione (GSH) in the liver of rats. Padma *et al.* (2016) have also recorded similar findings that *Tinospora cordifolia*, with its antioxidant effect, offered cytoprotection against cadmium-induced toxicity in kidneys by restoring the altered cellular antioxidants and renal markers in wistar rats. It, therefore, becomes evident that manifest its protective effect on BPA induced apoptosis by elevating the activity of antioxidants.

CONCLUSION

Hence, it becomes obvious that BPA manifest its effect through inducing oxidative stress as most of the plasticizers and environmental toxicant does. Ameliorative efficacy of Vitamin E and *Tinospora cordifolia* attenuate BPA induced testicular damage. This effect is attributed to free radicals scavenging the ability of Vitamin E and *Tinospora cordifolia* by inhibiting lipid peroxidation, strengthening the cellular antioxidant pool.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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