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# Enhanced oxidative stress and immunotoxicity due to *in vitro* exposure of captan in chicken lymphocytes

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**Abstract**
Chemical pesticides have become an integral part of modern agriculture industry though not without deleterious effects on human/ animals health. Captan is a commonly used fungicide belonging to the chemical group sulfenimide. There are reports indicating its inhibitory role in cellular DNA synthesis in herring sperm DNA and human cellular DNA, suggesting a possible mutagenic consequence. It was surprising to note that, though captan is a popular fungicide in use since early sixties, practically no information is available on *in vitro* immunotoxic effects of captan. Present communication reports oxidative stress and immunotoxic effects of low level dose of captan on chicken lymphocytes cell culture system using nitric oxide estimation and Lymphocyte proliferation assay. Captan treatment led to enhanced oxidative stress on estimating the nitric oxide level. It also displayed a significant decrease in B and T cell blastogenesis in comparison to control cells and thus found to be immunotoxic even at low level.

**Keywords:** Captan, immunotoxicity, oxidative stress, chicken lymphocytes, nitric oxide assay

## 1. Introduction
Pesticides are widely distributed and used in agriculture, animal husbandry and public health operations throughout the world. Captan [2-(trichloromethylsulfanyl)-3a,4,7,7a-tetrahydroisoindole-1,3-dione] is a fungicide belonging to the chemical group sulfenimide, used on fruits, vegetables, and ornamental plants sine long. Captan is the most frequently used fungicide on apple and pear trees in many countries. Several studies have indicated that captan is carcinogenic in rats and mice [1]. Captan has been adequately tested for genotoxicity in a range of assays, which demonstrate that it is mutagenic and clastogenic [2, 3]. This pesticide has been classified as potentially carcinogenic to humans by the European Community; it belongs to group 3 of the International Agency for Research on Cancer (IARC) classification (that is, sufficient evidence to establish the mutagenicity of captan in cellular systems, but insufficient data to establish its mutagenicity in mammals) [2, 4]. In short, captan is reported to induce immunomodulatory, mutagenic and teratogenic effects [2, 3, 5, 6].
Captan has been found to reduce cloning ability and produce DNA single-stranded breaks in the *in vitro* cultured human foreskin fibroblasts. Captan also inhibits cellular DNA synthesis and forms stable adducts in herring sperm DNA and human cellular DNA, suggesting a possible mutagenic consequence [7]. It was surprising to note that, though captan is a popular fungicide in use since early sixties, practically no information is available on *in vitro* immunotoxic effects of captan. The NOEL dose of captan is well documented in the literature [8, 9]. The pesticide dose selected for the present study was NOEL/$10^3$ which was found to be suitable for the *in vitro* studies in avian lymphocyte cell culture system. Present communication reports oxidative stress, immunosuppressive effects and apoptosis of low level dose of captan on chicken lymphocytes cell culture system using nitric oxide estimation, Lymphocyte proliferation assay.

## 2. Materials and Methods
### 2.1 Chicken lymphocytes
Chicken spleens were collected from healthy birds from a local slaughter house, and lymphocytes were isolated under laminar air flow as per standard procedure [10]. Lymphocytes

were separated through density gradient centrifugation (Histopaque 1077, Sigma) as per the method described by Rose and Friedman [11].

**2.2 Cell viability assay**

Percentage cell viability was determined by 0.1 per cent trypan blue dye exclusion test using haemocytometer [12] and final cell count was adjusted to $10^7$ cells/ ml in RPMI-1640 medium and made into one ml aliquots in eppendorf tubes and cells were pelleted by centrifugation at 1400 rpm for 10 min.

**2.3 Pesticide treatment**

Commercial preparation of captan was purchased from the local market and it's thousand times diluted NOEL (12.5 mg/ kg body weight) dose in RPMI- 1640 medium (Hi – Media, India) was used for the *in vitro* exposure of avian lymphocytes for two hours at 37ºC. After incubation cells were washed twice and finally suspended in 1 ml of RPMI-1640 medium supplemented with 10% FCS (Sigma, USA).

**2.4 Lymphocyte proliferation assay (LPA)**

LPA or B and T cell blastogenesis assay was carried out as per the method described by Rai-el-Balhaa *et al.* [13]. Concanavalin-A (ConA) (Sigma, USA) was used as a T cell mitogen whereas lipopolysaccharide (LPS) (Sigma, USA) as a B cell mitogen at a concentrations of 5 µg/ml, each, in RPMI-1640 medium. Flat bottom 96 well tissue culture plates (Corning, USA) were used for the assay. Each well was seeded with 200µl of lymphocytes suspension and the plate was incubated in a $CO_2$ incubator at $37^0$C. After 68 hours post cell seeding, the media was removed from each well carefully and 20µl of MTT (5mg/ml stock) in 200µl of media was added into each well and further incubated for 4 hours in the dark at $37^0$C in a $CO_2$ incubator. After incubation, dark blue formazan crystals were solubilized with 200µl of DMSO and absorbance was measured at 570 nm using computerized Micro Scan ELISA Reader. Survival rate was calculated from the relative absorbance at 570 nm and expressed as the percentage of control.

**2.5 Statistical analysis**

Analysis of variance (ANOVA) and student's t-test were used to estimate significant difference between control and treated cells. The values were expressed as mean delta Optical Density ± standard error (mean Δ OD± SE). Student t-test was employed for comparing the mean ODs [14].

**2.6 Oxidative Stress Assay**

Macrophages were isolated from spleen on the basis of their adherent properties [15]. Captan treated and control cells were seeded in 24-well culture plates. Cells were then incubated at 37ºC in 5% $CO_2$ for 4 hrs to allow the adherence of macrophages. After incubation, cells were washed vigorously four times with DMEM to remove non-adherent cells. These cells were incubated at 37ºC in a $CO_2$ incubator in the presence of LPS (Sigma) at a concentration of 5 µg/ml. Nitric oxide (NO) production by macrophages in the medium was measured by microplate assay method [16]. The standard curve to calculate the NO production was prepared using different dilutions of $NaNO_2$.

**3. Results**

**3.1 Lymphocyte proliferation assay (LPA)**

The *in vitro* exposure of avian lymphocytes to NOEL/$10^3$ dose of captan, showed a sharp reduction in B cell blastogenesis in the presence of B cell mitogen LPS as compared to the control cells (Table 1 and Fig. 1). In case of T lymphocytes proliferation, captan treated avian lymphocytes showed significant decrease in proliferation in the presence of mitogen ConA (Table 2 and Fig. 2).

**3.2 Oxidative stress assay**

As illustrated in the Table 3 and Fig.3, captan treated cells showed the more NO production as compared to the control with mean NO concentrations of 62.49 ± 0.492.

**4. Discussion and conclusions**

The acute toxicity of many pesticides used is well known and poisoning cases often documented. In contrast, much less is known about longer-term impacts on different systems of the human/ animal body including the nervous, hormone, reproductive and immune systems. Captan showed down regulation in B & T cell proliferation in avian lymphocyte cell culture system in the presence of respective mitogens. It was surprising to note that, though captan is a popular fungicide in use since early sixties and has been regularly evaluated [17], practically no information is available on *in vitro* immunotoxic effects of captan. Pilinskaia [18] investigated the mutagenic effects of fungicide captan and benomyl in the culture of human peripheral lymphocytes *in vitro* with and without metabolic activation and observed that the captan did not manifest cytogenetic activity in any experimental variance, while benomyl induced colchicines-like and weak clastogenic effects in the concentration 10 µg/ml in the presence of microsomal activating mixture only. Genotoxic studies on captan in *in vitro* system have shown its ability to degrade mammalian and fish DNA with possible mutagenesis [7]. Folpet and captan are fungicides show genotoxicity because of their chemical reaction with thiols. Data shows that these compounds have *in vitro* mutagenic activity but are not genotoxic *in vivo*. This dichotomy is primarily due to the rapid degradation of folpet and captan in the presence of thiol-rich matrices typically found *in vivo* [19]. captan induced dose- and time-dependent cytotoxicity and lipid peroxidative in isolated rat hepatocytes [20].

Present study showed enhanced oxidative stress through NO estimation in captan treated cells. There is a clearly established relationship between ROS/ free radicals and apoptosis [21-23]. Since ROS/ free radical intermediates mediate many immune cell functions and apoptosis has been established in immune cell populations, it is likely these two events could arise simultaneously during certain chemical exposures [24, 25]. Captan demonstrated limited induction of SCE in human lymphocytes *in vitro* because of toxic levels of either the fungicide or solvent used in the study [26]. A moderate suppression of PMA/Ionomycin induced GFP expression from IL-2, IFN-γ, IL-4 and IL-10 reporter jurkat T cell lines was obtained on captan treatment confirming immunosuppressive action of this compound [27]. The effect of short-term oral administration of captan, on the immune response was studied in rats and mice [6]. The SRBC-antibody formation, lymphoblastic stimulation of spleenic cells by PHA and by LPS was depressed in both species after 14 days of captan treatment. These results pointed out a clear suppressive effect of captan on the immune response of the animals which are consistent with present findings. Analyzing the outcome of the present studies it can be concluded that *in vitro* exposure of low level dose of captan caused oxidative stress and immunotoxicity in avian lymphocytes.

**Table 1:** *In vitro* effects of captan on NO concentration (µM/ml) in mononuclear cells

| S. No | Treatments | Mean Conc. ± S.E.** | Percentage change |
|---|---|---|---|
| 1. | Control | 39.04 ± 1.233 | – |
| 2. | Captan | 62.49 ± 0.492 | **+60.07** |
| CD at 1% = 3.912 CD at 5% = 2.792 | | | |

**Table 2:** *In vitro* effects of captan and cow urine on B cell blastogenesis in avian lymphocytes

| S. No. | Treatments | Mean Δ O.D. ± S.E.** | Percentage change |
|---|---|---|---|
| 1. | Control | 0.266 ± 0.010 | – |
| 2. | Captan | 0.034 ± 0.002 | -87.22 |
| CD at 1% = 0.047 CD at 5% = 0.033 | | | |

**Table 3:** *In vitro* effects of captan on T cell blastogenesis in avian lymphocytes

| S. No. | Treatments | Mean Δ O.D. ± S.E.** | Percentage change |
|---|---|---|---|
| 1. | Control | 0.286 ± 0.007 | – |
| 2. | Captan | 0.035 ± 0.002 | -87.76 |

CD at 1% = 0.041 CD at 5% = 0.029

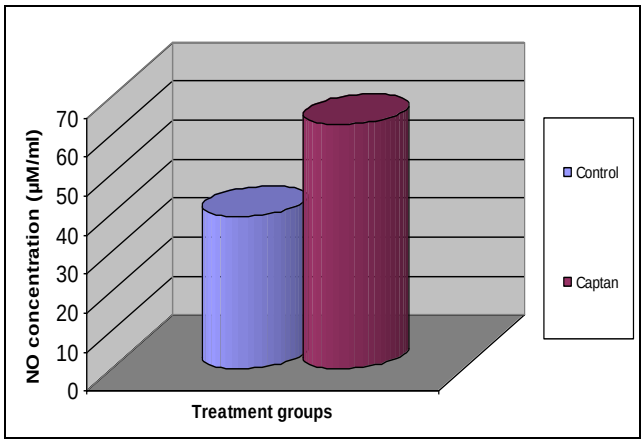


**Fig 1:** Effects of captan on nitric oxide (NO) concentration in mononuclear cells

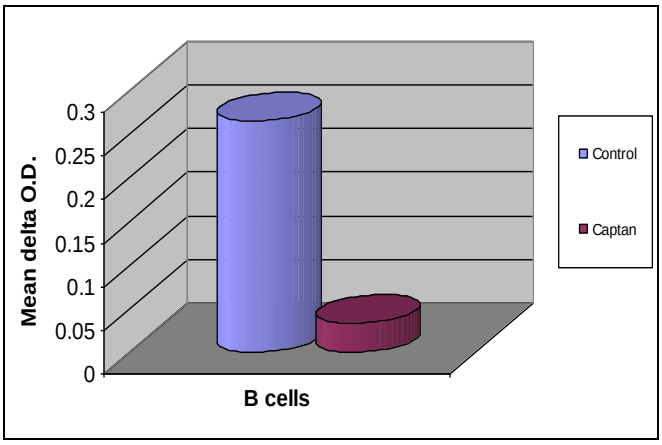


**Fig 2:** Effects of captan on B cell blastogenesis in avian lymphocytes

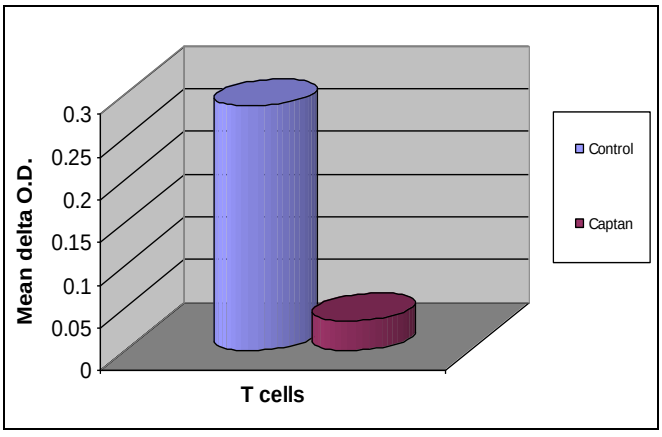


**Fig 3:** Effects of captan T cell blastogenesis in avian lymphocytes

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