SUMMARY AND CONCLUSIONS
The health implications of immune dysfunctions are increased risk of infectious diseases, development of neoplasia, autoimmune disorders and allergies. In recent years, several studies indicate that adverse effects on the immune system result from occupational, inadvertent or therapeutic exposure to drugs, environmental chemicals and, in some instances, biological materials. The interaction of toxic chemicals with man or animals is dependent on chemical structure, exposure factors, age and nutritional status of the organism. Therefore, a comprehensive safety assessment of chemicals, which are consumed either through habit or for therapeutic use is necessary. Such a study is needed for arecoline, a major betel nut alkaloid, since it is consumed by people in oriental countries through their chewing habits and for its anthelmintic and cholinergic therapeutic effects. Apart from many epidemiological studies, a number of experimental studies indicate carcinogenic, mutagenic and teratogenic abilities of arecoline. Since these potencies are also associated with undesirable effects on the immune system, preliminary and rudimentary studies conducted earlier in our laboratory and elsewhere showed possible immunosuppressive nature of arecoline. It was not known from those studies, the time-response and dose-response relationship, the possible way by which it affects immune organs as well as immune response.
and phase specific alterations. Therefore, the present investigation is an attempt in those directions employing a battery of test assays to comprehensively evaluate arecoline action on immune system and on immune response to T-cell dependent antigen, Sheep Red Blood Cell (SRBC). These tests include selected pathotoxicological and immunological parameters given below:

I. **Pathotoxicological Parameters**

Changes in body/organ weights; histology of lymphoid as well as other organs; blood cell counts, and clinical biochemistry.

II. **Immunological Parameters**

1) Cell mediated and indirect immunity
   - Delayed hypersensitivity reactions to SRBC
   - Con A induced lymphocyte proliferative response
   - Resistance to endotoxin shock.

2) Humoral immunity
   - Primary and secondary immune responses to SRBC as evaluated by plaque forming cell assay and the circulating antibody titers
   - Pokeweed mitogen induced lymphocyte proliferative response.
Swiss albino mice, Balb/c mice and Wistar albino rats were used as animal models. The dose regimens (5, 10 and 20 mg/kg body weight) used in the present study were based on L.D.50 value of arecoline.

Arecoline was administered subcutaneously to experimental mice for a period ranging from one to three weeks, treated daily. At the termination of the specified treatment periods, both pathotoxicological and immunological parameters were studied as mentioned above. In order to evaluate phase specific immunological effects of arecoline, mice were treated with arecoline at various times following immunization.

The following are some of the important findings from in vivo and in vitro studies:

I. Pathotoxicological Study

In vivo experiments:

1) Acute subcutaneous L.D.50 of arecoline in both sexes of Swiss albino mice was determined to be 97 mg/kg bw. The oral L.D.50 values for male and female mice were 371 and 309 mg/kg bw, respectively, and the intraperitoneal L.D.50
values for male and female mice were 120 and 109 mg/kg bw, respectively.

2) Chronic arecoline treatment, at various doses and after different time intervals, revealed a significant reduction in growth rate (body weight) of animals only at 20 mg/kg bw, the lower doses showing less pronounced effects.

3) A reduction in thymus weight and body weight/thymus weight ratio was observed, in a dose-dependent manner, with significant reduction at 20 mg/kg bw dose; no significant change was seen at 5 mg/kg bw dose.

4) The other organs, such as spleen, mesenteric lymph nodes, liver and kidney showed a moderate reduction in their weight according to the dose of arecoline. A mild increase in the weight of adrenals was seen at a 20 mg/kg bw. However, such changes seen in those organs were not consistent with the duration of treatment.

5) At 20 mg/kg bw dose of arecoline, thymus exhibited cortical atrophy with severe reduction in the cellularity and mild reduction in the medullary regions. Spleen showed less cellularity in the
periarteriolar lymphatic sheaths with inconspicuous germinal follicles. Mesenteric lymph nodes showed a mild decrease in cellularity of the medulla and paracortex. The adrenals were characterized by marked hypertrophy of zona fasciculata. These changes were relatively less at 10 mg/kg bw. At 5 mg/kg bw treatment there was no appreciable alteration. At any dose, the changes were not dependent on the duration of treatment. Histologically, liver and kidneys did not differ from controls.

6) RBC and WBC counts were reduced in a dose-dependent manner, with maximum reduction at 20 mg/kg bw and no effect at 5 mg/kg bw.

7) After arecoline treatment, total protein, albumin, glucose, acid phosphatase and hemoglobin concentrations in blood/serum did not show any appreciable change. SGOT and SGPT levels showed changes according to the dose of arecoline treatment.

8) In concordance with the zona fasciculata hypertrophy of adrenals, corticosterone levels in serum
increased, in a dose-dependent manner, with no appreciable alteration at low dose.

9) In thymus, spleen and mesenteric lymph nodes, treatment of arecoline at 20 mg/kg bw, affected cellularity, without causing much influence on cell viability. These changes were much less at 10 mg/kg bw dose; no change was seen at 5 mg/kg bw dose.

**In vitro experiment:**

1) A biphasic response in oxygen consumption of rat thymocytes was demonstrated in the presence of arecoline at various concentrations. With increasing concentration of arecoline, there was a progressive increase in oxygen consumption, reaching a maximum value at $10^{-5}\text{M}$, whereas, at $10^{-3}\text{M}$, oxygen consumption was reduced. The viability of rat thymocytes was not affected during the incubation period, at any concentration of arecoline. Exogenously added substrates such as glucose, pyruvic acid and lactic acid eliminated the fall in the oxygen consumption induced by $10^{-3}\text{M}$ arecoline.
II. Immunological Parameters

Cell Mediated Immunity

In vivo experiments:

1) Delayed type hypersensitivity (DTH) reactions to SRBC, evaluated in both sexes of mice, treated with 20 mg/kg bw dose of arecoline, were significantly suppressed, as compared to that of controls. At 10 mg/kg bw, there was a moderate reduction, with no appreciable change at 5 mg/kg bw dose. The effects were not dependent on the duration of treatment.

2) Treatment of arecoline continuously for 4 days following SRBC immunization showed significant suppression in DTH reactions at 10 and 20 mg/kg bw doses. When treated with 20 mg/kg bw arecoline, 12 h after immunization, there was significant reduction in DTH reactions. Moderate reduction in response was observed with 10 mg/kg bw arecoline given at 12 h after immunization. The treatment with 5 mg/kg bw arecoline did not alter the DTH reactions.

3) The DTH response obtained in aged female mice (∼15 month old) was lower than that observed with
young adult mice. Arecoline treatment for a week reduced the response in a dose-dependent manner, with maximum suppression at 20 mg/kg bw. There was no appreciable alteration at 5 mg/kg bw arecoline.

4) Recovery experiments in mice revealed that arecoline mediated effects are of reversible nature.

In vitro experiments

5) Both dose-dependent and time-dependent cytotoxic effects of arecoline were noticed when spleen cells were incubated with varying concentrations of arecoline.

6) Spontaneous proliferative response of spleen cells as evaluated by $^3$H-thymidine incorporation was suppressed at $10^{-4}$M arecoline, whereas, at $10^{-8}$M - $10^{-5}$M it did not appreciably affect the response.

7) Concomitant exposure of arecoline at $10^{-6}$ and $10^{-5}$ M with concanavalin A, a mitogen, significantly suppressed the $^3$H-thymidine incorporation.

8) Arecoline administration at 12 h after con A stimulation of spleen cells showed marked
suppression of $^3$H-thymidine incorporation. These changes were slightly more pronounced than the results obtained with concomitant exposure.

9) Concomitant exposure of arecoline at $10^{-6}$ and $10^{-5}$ M with con A appreciably suppressed Interleukin-2 (IL-2) production.

10) Concomitant exposure of arecoline with IL-2 did not alter $^3$H-thymidine incorporation in the IL-2 dependent cytolytic T-lymphocytes, except at $10^{-4}$M where even spontaneous incorporation was also affected.

11) Arecoline treatment did not appreciably alter the host resistance to endotoxin shock.

**Humoral Immunity**

**In vivo experiments**

1) Arecoline treatment showed a dose-dependent suppression of primary (IgM) antibody forming cells to SRBC with a maximum reduction at 20 mg/kg bw dose.

2) Antibody titers to SRBC, and total IgG, showed reduction in arecoline treated mice with
significant suppression at 10 and 20 mg/kg bw. The effect was not duration-dependent.

3) Like primary humoral immune response, the secondary immune response was also decreased after arecoline treatment. The responses obtained with 10 and 20 mg/kg bw were marked.

4) Treatment of arecoline at 10 and 20 mg/kg bw, for 4 days following SRBC immunization, showed dose-dependent suppression of primary immune responses. The animals which received 20 mg/kg bw arecoline at 12 h after immunization showed significant reduction, whereas, moderate reduction was evident at 10 mg/kg bw dose. Arecoline treatment at 5 mg/kg bw did not alter the response.

5) The primary antibody response obtained in aged female mice (≈ 15 month old) was lower than that in young adult mice. Arecoline treatment for a week reduced the response in a dose-dependent manner with maximum suppression at 20 mg/kg bw dose.

6) Recovery experiments in mice revealed that arecoline mediated effects are of reversible nature.
In vitro experiments

1) Concomitant exposure of only $10^{-5}$ M arecoline with Pokeweed mitogen significantly suppressed $^3$H-thymidine incorporation. This observation suggests that arecoline affects Pokeweed mitogen-induced response less than that induced by con A.

2) Administration of $10^{-6}$ - $10^{-5}$M arecoline, at 12 h after Pokeweed mitogen stimulation of spleen cells showed significant suppression of $^3$H-thymidine incorporation. These changes were slightly more than the results obtained with concomitant exposure.

CONCLUSIONS

Results obtained in the present study demonstrate the chemically-induced immunomodulation by arecoline at subtoxic dose levels. The effects of arecoline on cell mediated and humoral immune functions are more clear than the effects on host resistance to endotoxin shock. It could be further stated that the cooperative phenomenon of T- and B-cells as such is affected due to arecoline treatment as T-dependent antigen was employed. The present and earlier studies so far conducted on immunotoxicity of arecoline deal with only T-cell dependent antigen. In order to delineate
the effect on cell mediated and humoral responses, it is necessary to assess the responses to T-independent antigens.

Further, the study clearly indicates that the effect of arecoline is dependent on dose rather than on the duration of treatment. Continuous treatment of arecoline is required for effective and sustained immunosuppression. Arecoline seems to affect the immune system both directly and indirectly. It depletes the number of cells in lymphoid organs without affecting cell viability. Arecoline seems to induce these changes indirectly by elevating the level of corticosterone. The suppressive effect of arecoline on mitogen-induced \textit{in vitro} proliferation and its interference with \textit{in vitro} oxidative metabolism of thymocytes (in addition to the very chemical nature of arecoline, i.e., cholinergic) indicate that possibly it can also affect the immune system directly. Thus, the immunomodulatory effect of arecoline seems to be through more than one mechanism, like many other chemicals which affect the immune system.

The sophistication and complexity of the immune system lends itself to be the most sensitive, and therefore, the most prominent body function to detect the harmful effects of chemicals and drugs. Further studies, therefore, are necessary to delineate arecoline induced immunomodulation, and its impact on public health.