CHAPTER IV

RESULTS

1. Determination of optimal cell density and lipopolysaccharide (LPS) concentration for J774A.1 cell stimulation

The objectives of this experiment were to select appropriate cell density and lowest concentration of LPS for stimulating J774A.1 macrophage cells to produce sufficient amounts of nitric oxide (NO). The results in NO concentration are shown in Figure 4.

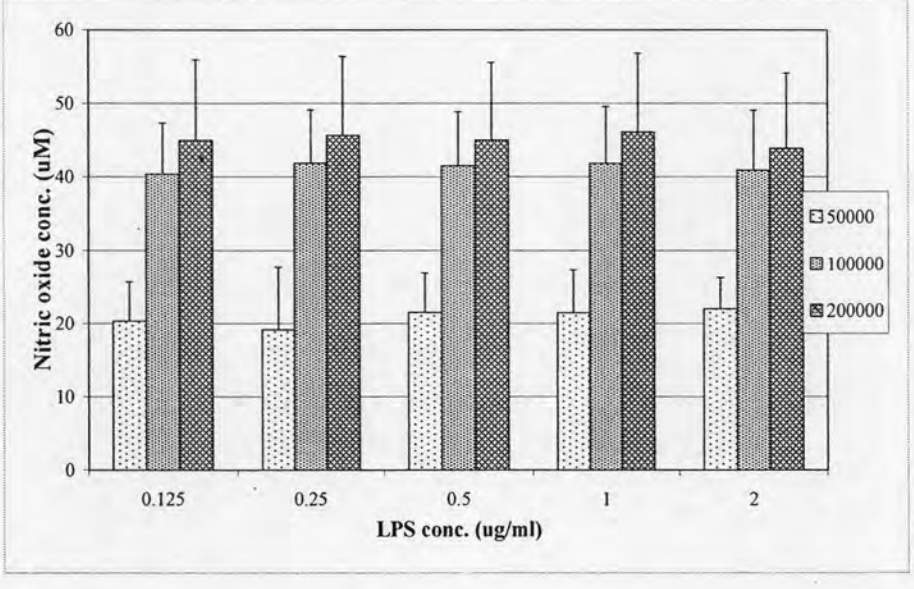

Figure 4: Effects of cell density and LPS concentration on NO production in J774A.1 cells. J774A.1 cells were seeded at different cell densities ($0.5x10^5$ to $2x10^5$ cells/ml). LPS concentration was between 0.125–2 μg/ml. The displayed values are mean ± S.D. (n=9).

At a specific cell density, increase in concentration of LPS from 0.125 to 2 μg/ml did not result in significant increase in NO production ($P>0.05$). On the contrary, LPS-induced NO production increased when the seeding density was increased from $0.5x10^5$ to $2x10^5$ cells/ml at all LPS concentrations. NO production at $0.5x10^5$ cells/ml was significantly lower than NO production seen with the other two seeding densities

($P<0.05$). However, there was no significant difference between the NO production at cell densities of $1x10^5$ and $2x10^5$ cells/ml ($P>0.05$). Thus, the lowest concentration of LPS (0.125 μg/ml) and the cell density of $1x10^5$ cells/ml were selected for further experiments.

2. Determination of optimal antioxidant concentrations for NO inhibition in LPS-stimulated J774A.1 cells

The aim of this experiment was to select appropriate concentrations of the two model antioxidants, namely *N*-acetylcysteine (NAC) and α-tocopherol (TOC) that would display significant inhibition on NO production in LPS-stimulated J774A.1 cells. A low percentage of viability (lower than 80%) was selected as the cutoff point for cytotoxicity.

2.1 Selection of TOC concentrations for NO inhibition in LPS-stimulated J774A.1 cells

NO production in LPS-stimulated J774A.1 cells in the presence of TOC was compared with that in response to LPS only. The percentage of NO inhibition at various TOC concentrations is illustrated in Figure 5. LPS-stimulated NO production by J774A.1 cells was significantly reduced when the cells were exposed to increasing concentrations of TOC from 0.5 to 1.0 mM ($P<0.05$, Appendix C-1). However, cytotoxicity of TOC dispersion was clearly seen at 1 mM (Figure 6). Therefore, TOC at 0.25 and 0.5 mM were selected for subsequent experiments to determine whether encapsulation of TOC in liposomes would increase its inhibitory effect on NO production.

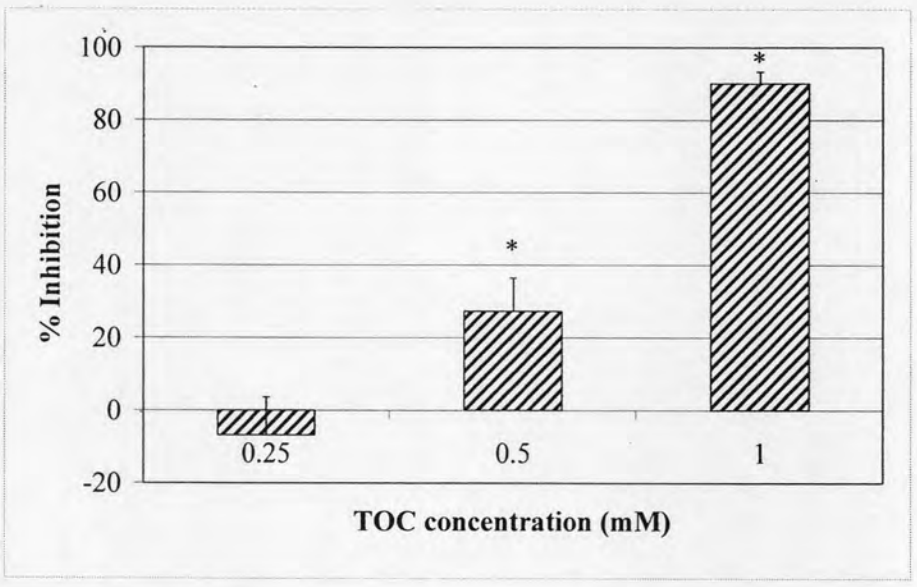

Figure 5: Effect of TOC concentration on NO production in LPS-stimulated J774A.1 cells. Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. The displayed values are mean ± S.D. (n=9).

* significantly different from the control ($P<0.05$)

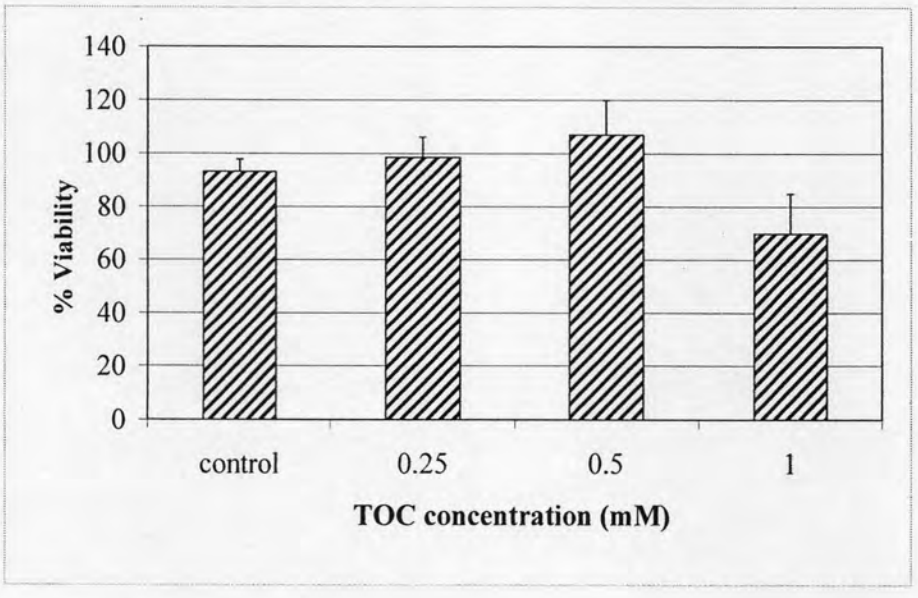

Figure 6: Effect of TOC concentration on cell viability of J774A.1 cells by MTT assay. Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. The displayed values are mean ± S.D. (n=9).

2.2 Selection of NAC concentrations for NO inhibition in LPS-stimulated J774A.1 cells

The percentage of NO inhibition at various NAC concentrations is displayed in Figure 7. The addition of NAC (≥ 10 mM) resulted in decreased NO production compared with the control ($P<0.05$, Appendix C-2). No cytotoxicity was seen in all the doses studied. The cell viability was not significantly different among all treatments with NAC and the control, as illustrated in Figure 8. Therefore, NAC concentrations at 10 and 20 mM were selected for subsequent experiments with liposomes.

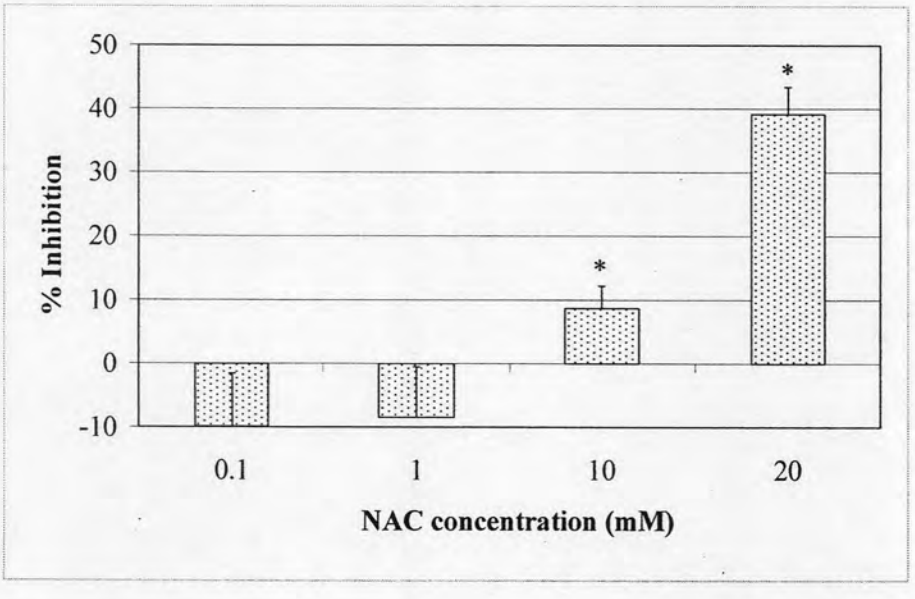

Figure 7: Effect of NAC concentration on NO production in LPS-stimulated J774A.1 cells. Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 µg/ml. The displayed values are mean ± S.D. (n=9).

* significantly different from the control ($P<0.05$)

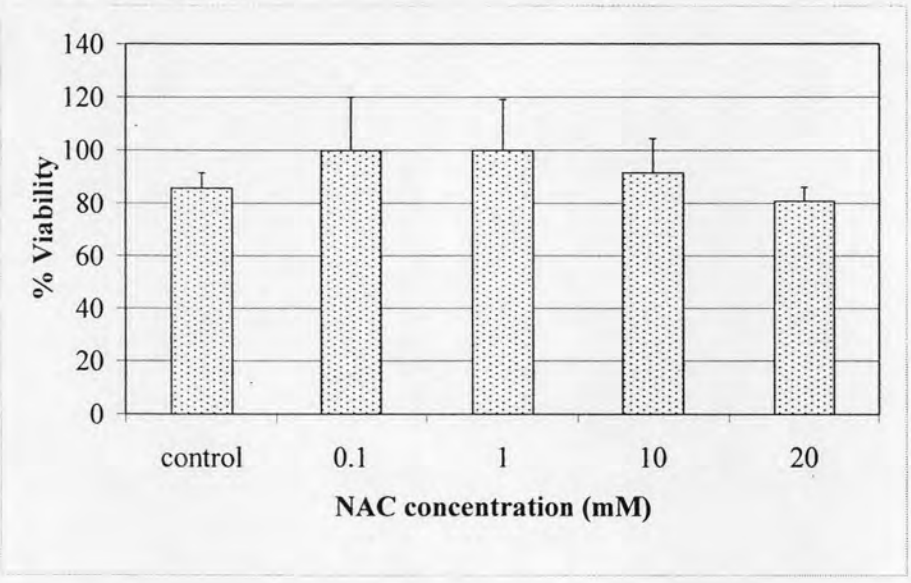

Figure 8: Effect of NAC concentration on cell viability of J774A.1 cells by MTT assay. Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. The displayed values are mean ± S.D. (n=9).

3. Effects of blank liposomes on NO production in LPS-stimulated J774A.1 cells

The aim of this experiment was to determine the effects of blank liposomes on NO production in J774A.1 cells. The viability of liposome-treated cells was determined to confirm that blank liposomes exerted no cytotoxic effect on J774A.1 cells.

3.1 Effect of blank PC/CH liposomes

Figure 9 illustrates inhibitory effect of blank neutral liposomes (PC:CH, 70:30) on LPS-stimulated NO production in J774A.1 cells at various total lipid concentrations (1-4 mg/ml). The inhibitory effect of blank neutral liposomes increased in a concentration dependent manner ($P<0.05$, Appendix C-3). No cytotoxicity was seen in all concentrations studied. The effect of blank PC/CH liposomes on cell viability is shown in Figure 10.

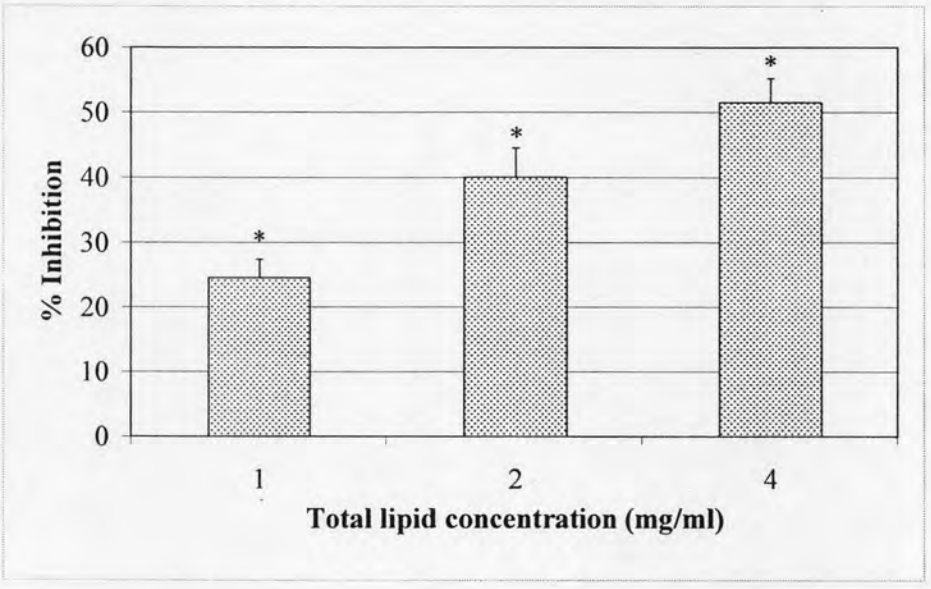

Figure 9: Effect of PC/CH liposome concentration on NO production in LPS-stimulated J774A.1 cells. Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. The displayed values are mean ± S.D. (n=9).

* significantly different from the control ($P<0.05$)

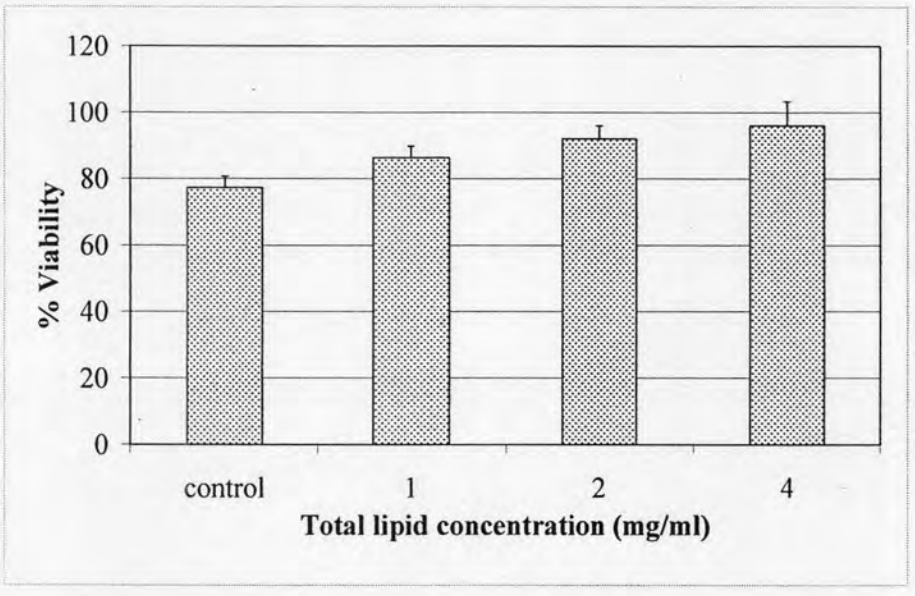

Figure 10: Effect of PC/CH liposome concentration on cell viability of J774A.1 cells by MTT assay. Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. The displayed values are mean ± S.D. (n=9).

3.2 Effect of blank PC/CH/DCP liposomes

Effect of PC/CH/DCP liposomes on LPS-stimulated NO production and cell viability in J774A.1 cells are shown in Figures 11-12. The PC/CH/DCP liposomes (PC:CH:DCP, 60:30:10) at all total lipid concentrations significantly inhibited NO production when compared to the control ($P<0.05$, Appendix C-4). However, there was no significant difference between the inhibitory effect of PC/CH liposomes and PC/CH/DCP liposomes at 4 mg/ml of total lipid concentration. No cytotoxicity was seen in all concentrations studies, similar to the result seen with the blank PC/CH liposomes.

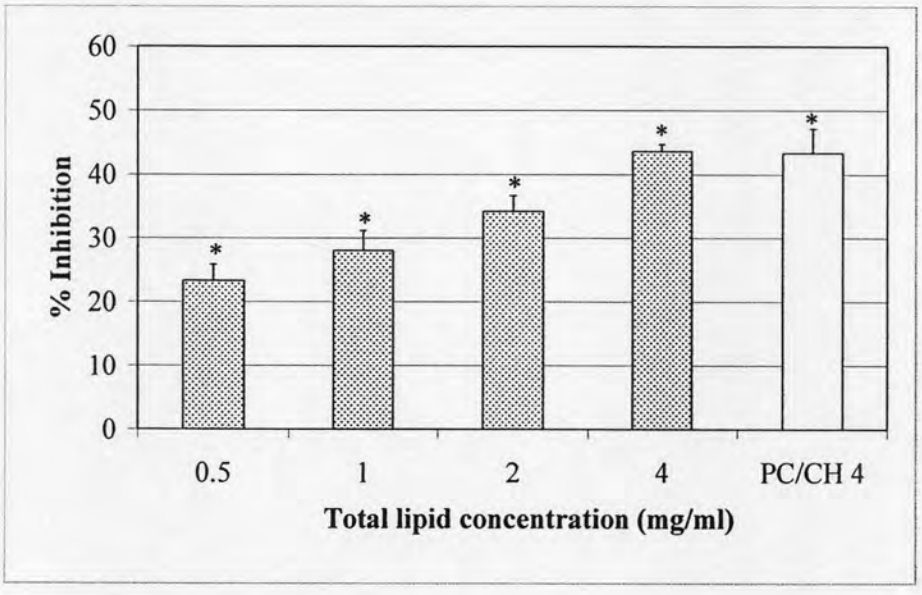

Figure 11: Effect of PC/CH/DCP liposome concentration on NO production in LPS-stimulated J774A.1 cells. Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. Solid columns represent PC/CH/DCP liposomes. Open column represents PC/CH liposomes at 4 mg/ml. The displayed values are mean ± S.D. (n=9). * significantly different from the control ($P<0.05$)

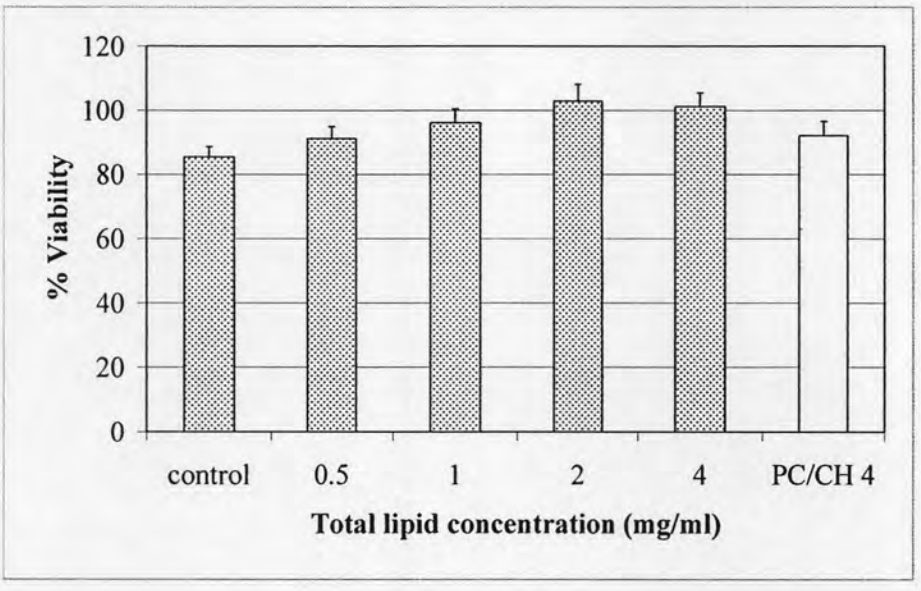

Figure 12: Effect of PC/CH/DCP liposome concentration on cell viability of J774A.1 cells by MTT assay. Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. Solid columns represent PC/CH/DCP liposomes and the control. Open column represents PC/CH liposomes at 4 mg/ml. The displayed values are mean ± S.D. (n=9).

3.3 Effect of blank PC/CH/PG liposomes

The effect of PC/CH/PG liposomes on NO production in LPS-stimulated J774A.1 cells is depicted in Figure 13. The inhibitory effect of blank PC/CH/PG liposomes on NO production was significantly seen at all total lipid concentrations ($P<0.05$, Appendix C-5). At 4 mg/ml total lipid, the magnitude of inhibition of blank PC/CH/PG liposomes is not significantly different from that of blank PC/CH liposomes ($P>0.05$, Appendix C-5). The effect of blank PC/CH/PG liposomes on cell viability was shown in Figure 14. No cytotoxicity was seen in all concentrations studied when compared to the control.

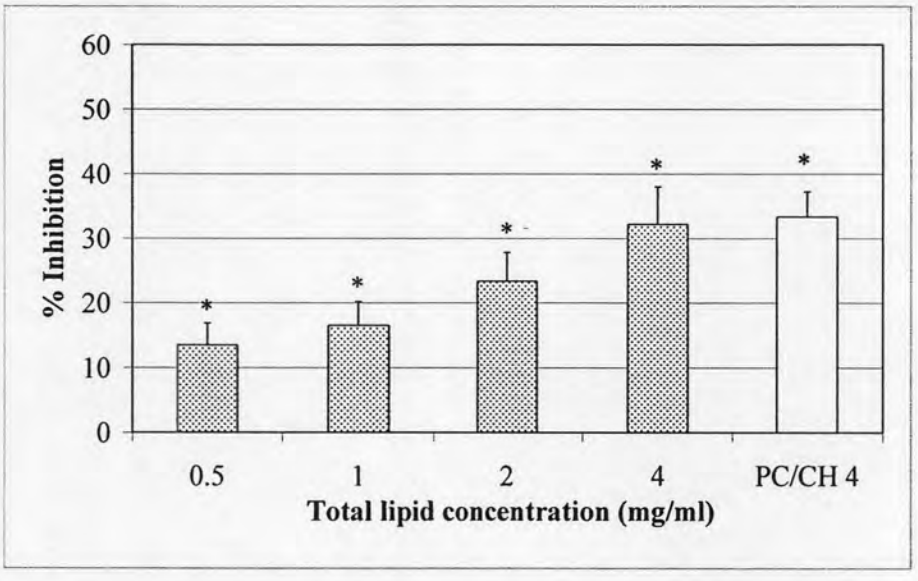

Figure 13: Effect of PC/CH/PG liposome concentration on NO production in LPS-stimulated J774A.1 cells. Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. Solid columns represent PC/CH/PG liposomes. Open column represents PC/CH liposomes at 4 mg/ml. The displayed values are mean ± S.D. (n=9). * significantly different from the control ($P<0.05$)

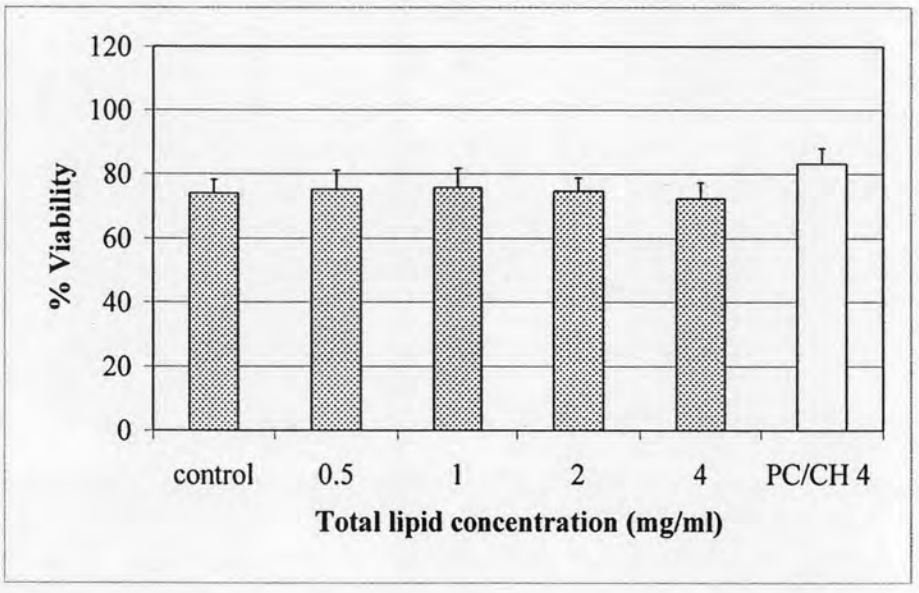

Figure 14: Effect of PC/CH/PG liposome concentration on cell viability of J774A.1 cells by MTT assay. Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. Solid columns represent PC/CH/PG liposomes and the control. Open column represents PC/CH liposomes at 4 mg/ml. The displayed values are mean ± S.D. (n=9).

3.4 Effect of blank liposomes without CH

At the total lipid concentration of 4 mg/ml, the percentages (mean ± S.D.) of inhibition of NO production in J774A.1 cells by PC, PC/DCP and PC/PG blank liposomes were 43.23 ± 12.38, 61.89 ± 8.53 and 51.75 ± 3.96 percent, respectively.

4. Effects of antioxidant-loaded liposomes on NO production in LPS-stimulated J774A.1 cells

4.1 Effect of TOC-loaded liposomes

The experiments were designed to determine the inhibitory effect of TOC-loaded liposomes with various lipid compositions (PC, PC/DCP and PC/PG) on NO production in LPS-stimulated J774A.1 cells. CH was omitted from all preparations containing TOC in order to facilitate incorporation of TOC into liposome bilayers. Two different concentrations of TOC were loaded into each liposome composition. All of the liposome formulations used in this study showed no cytotoxicity in J774A.1 cells (Appendices D-1 to D-3).

The results of TOC-loaded PC liposomes are illustrated in Figure 15. The results indicated that the TOC-loaded PC liposomes at both TOC loading concentrations had no significant difference in inhibitory effect on NO production when compared to the blank PC liposomes ($P>0.05$, Appendix C-6). TOC dispersion at 0.5 mM gave comparable result to the liposomes.

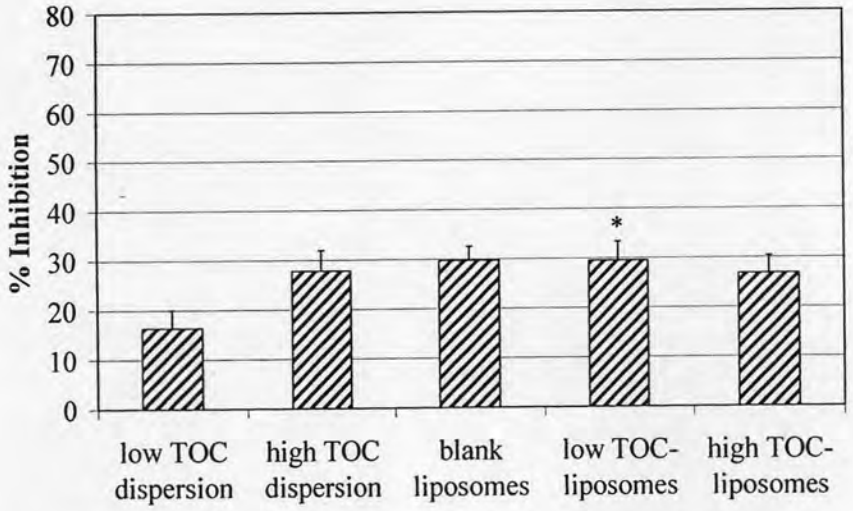

Figure 15: Inhibitory effect of TOC-loaded PC liposomes on NO production in LPS-stimulated J774A.1 cells. TOC was used at 0.25 mM (low TOC) and 0.5 mM (high TOC). Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. The values displayed are mean ± S.D. (n=6).

* significant difference between liposomes and the corresponding solution ($P<0.05$)

The effects of TOC loading in negatively charged liposomes on NO production are depicted in Figures 16 and 17. Loading of TOC in PC/DCP liposomes showed a trend of decrease in inhibitory effect compared with blank PC/DCP liposomes. However, the decrease was statistically significant only at the lower TOC loading concentration (0.25 mM) ($P<0.05$, Appendix C-7). Though the inhibitory effect was decreased when compared to blank liposomes, the effect seen was still higher than the effect of TOC dispersion at both TOC loading concentrations ($P>0.05$). On the contrary, there was no statistically significant difference between TOC-loaded PC/PG liposomes and blank PC/PG liposomes ($P>0.05$, Appendix C-8). The inhibitory effect of TOC-loaded PC/PG liposomes was significantly higher than the effect of TOC dispersion at the same TOC concentration ($P<0.05$, Appendices C-7 and C-8). Furthermore, the two different concentrations of TOC showed no significant effect in both types of negatively charged liposomes ($P>0.05$).

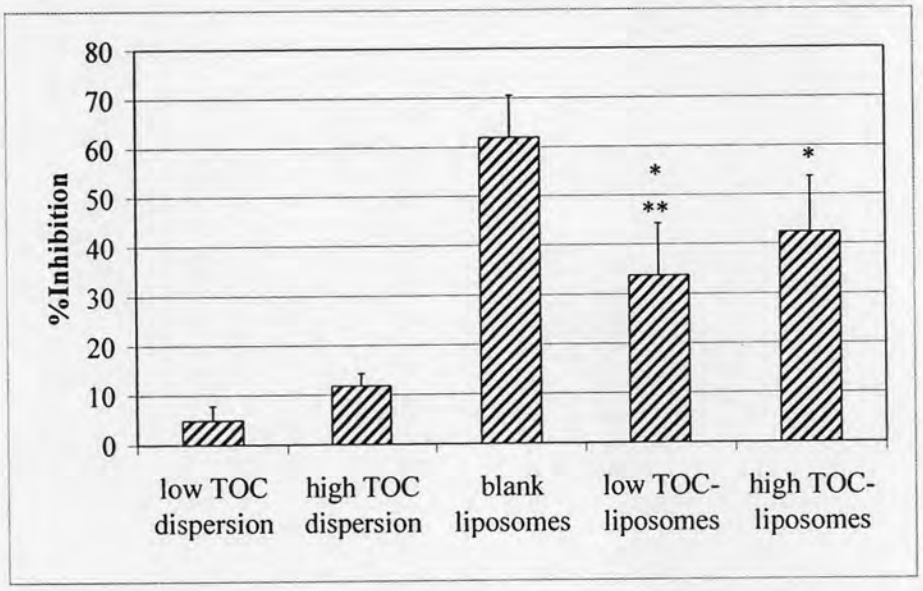

Figure 16: Inhibitory effect of TOC-loaded PC/DCP liposomes on NO production in LPS-stimulated J774A.1 cells. TOC was used at 0.25 mM (low TOC) and 0.5 mM (high TOC). Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. The values displayed are mean ± S.D. (n=6).

* significant difference between liposomes and the corresponding solution ($P<0.05$)

** significantly different from blank liposomes ($P<0.05$)

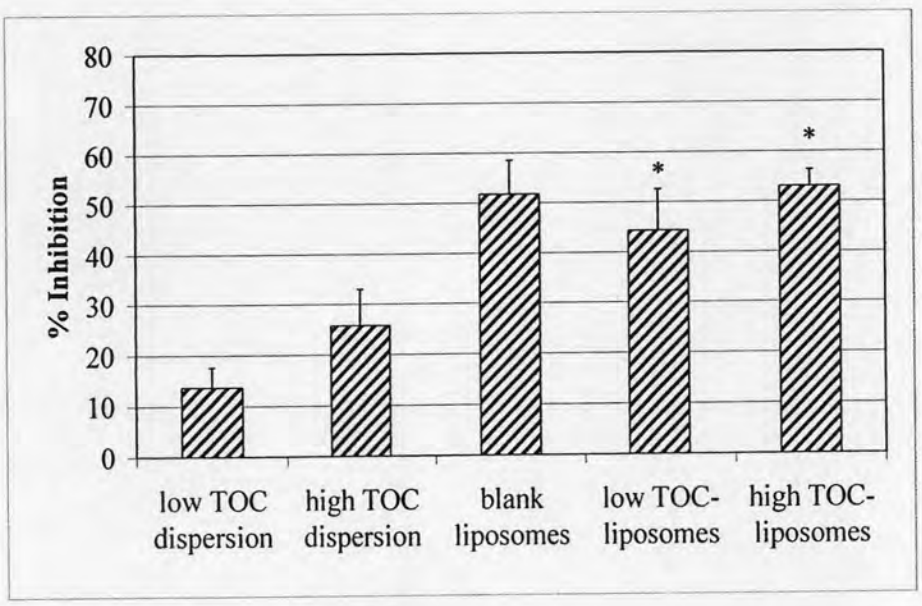

Figure 17: Inhibitory effect of TOC-loaded PC/PG liposomes on NO production in LPS-stimulated J774A.1 cells. TOC was used at 0.25 mM (low TOC) and 0.5 mM (high TOC). Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. The values displayed are mean ± S.D. (n=9).

* significant difference between liposomes and the corresponding solution ($P<0.05$)

4.2 Effect of NAC-loaded liposomes

The experiments were designed to determine the inhibitory effect of NAC-loaded liposomes with various lipid compositions (PC/CH, PC/CH/DCP and PC/CH/PG) on NO production in LPS-stimulated J774A.1 cells. Two different concentrations of NAC were loaded into each liposome composition. The experimental design was similar to that of TOC-loaded liposomes.

The result of NAC-loaded PC/CH liposomes are shown in Figure 18. NAC-loaded PC/CH liposomes at both of NAC loading concentrations showed a significant decrease in the inhibitory effect on NO production when compared with the blank PC/CH liposomes ($P<0.05$, Appendix C-9). NAC solution at 20 mM gave a comparable result to NAC-loaded PC/CH liposomes at the same NAC concentration. On the contrary, NAC-loaded PC/CH liposomes at 10 mM NAC concentration inhibited NO production to a higher extent than 10 mM NAC solution did. No cytotoxicity was seen with NAC-loaded PC/CH liposomes at both of NAC concentrations (Appendix D-4). In contrast with neutral liposomes, the NAC-loaded negatively charged (PC/CH/DCP and PC/CH/PG) liposomes at both NAC concentrations displayed severe cytotoxic effect on J774A.1 cells (Figures 19 and 20). An attempt to decrease liposome concentration was performed on NAC-loaded PC/CH/DCP liposomes. However, severe cytotoxicity was also seen when the liposome concentration was reduced to 1 mg/ml total lipid, where cell viability was only 73 % of the control. Therefore, the results of subsequent experiments on NO production with negatively charged liposomes were excluded from the study.

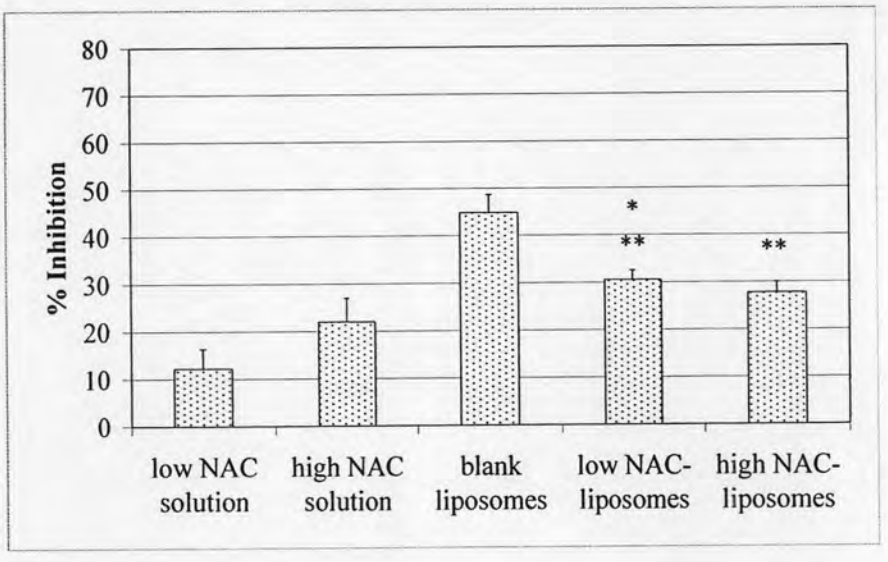

Figure 18: Inhibitory effect of NAC-loaded PC/CH liposomes on NO production in LPS-stimulated J774A.1 cells. NAC was used at 10 mM (low NAC) and 20 mM (high NAC). Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 µg/ml. The values displayed are mean ± S.D. (n=9).

* significant difference between liposomes and the corresponding solution ($P<0.05$)

** significantly different from blank liposomes ($P<0.05$)

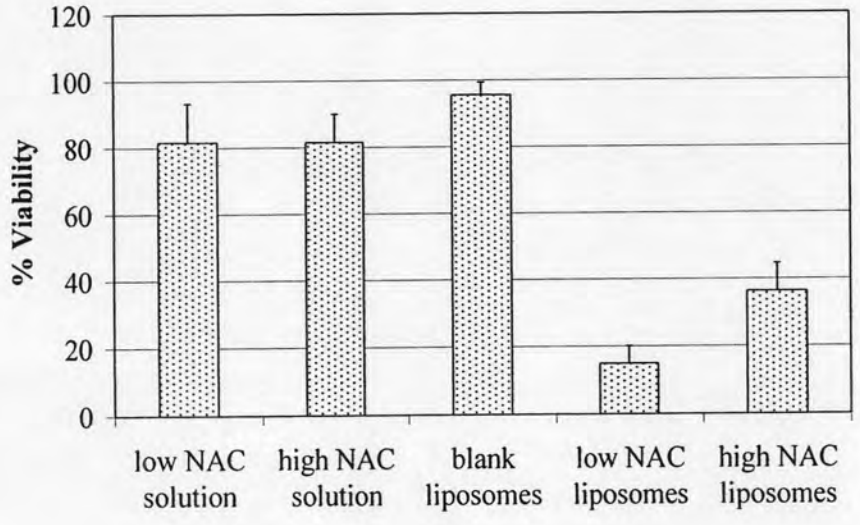

Figure 19: Effect of NAC-loaded PC/CH/DCP liposomes on cell viability of J774A.1 cells by MTT assay. NAC was used at 10 mM (low NAC) and 20 mM (high NAC). Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 µg/ml. The displayed values are mean ± S.D. (n=6)

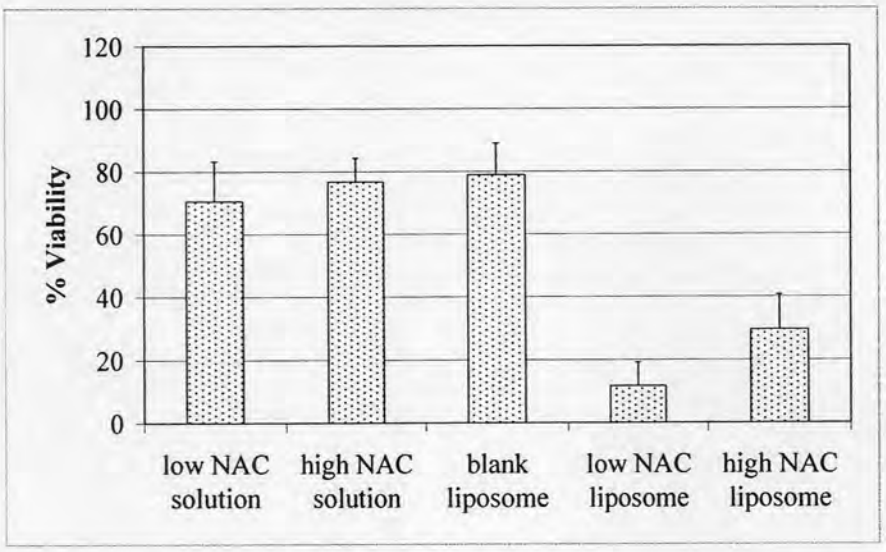

Figure 20: Effect of NAC-loaded PC/CH/PG liposomes on cell viability of J774A.1 cells by MTT assay. NAC was used at 10 mM (low NAC) and 20 mM (high NAC). Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. The displayed values are mean ± S.D. (n=6)

5. Effect of co-incubation of blank liposomes and antioxidants on NO production in LPS-stimulated J774A.1 cells

The aim of this experiment was to determine the inhibitory effect of co-incubation of antioxidant solution and blank liposomes with various lipid compositions on NO production in LPS-stimulated J774A.1 cells. The results would indicate whether the effects seen in the previous experiments were consequences of incorporation of the model antioxidant molecules into liposome structures. Two different concentrations of each antioxidant solution were used with each type of blank liposomes. All of the conditions used in this study exerted no significant cytotoxicity on J774A.1 cells (Appendices D-5 to D-10).

5.1 Effect of co-incubation of blank liposomes and TOC dispersions

The trend in inhibitory effect of co-incubation of each blank liposome formulation and TOC dispersion was similar to the inhibitory effect of TOC-loaded liposomes (Figures 21-23). In general, co-incubation of TOC with blank liposomes resulted in higher inhibitory effect than the effect of TOC dispersion. The effect seemed to be reduced when compared to the treatment with blank liposomes only. However, the differences were not significant, except for that of PC/PG liposomes (Appendices C-10 to C-12).

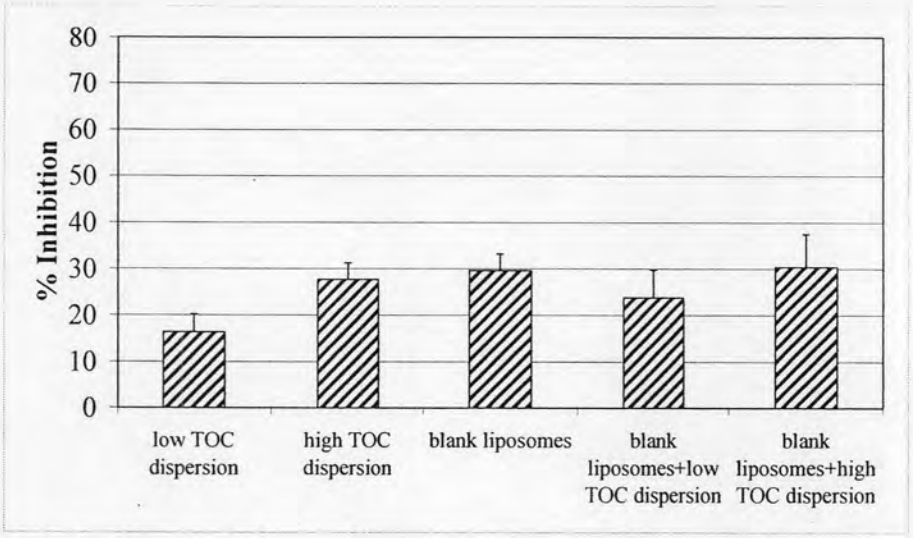

Figure 21: Inhibitory effect of co-incubation with blank PC liposomes and TOC dispersion on NO production in LPS-stimulated J774A.1 cells. TOC was used at 0.25 mM (low TOC) and 0.5 mM (high TOC). Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. The values displayed are mean ± S.D. (n=6).

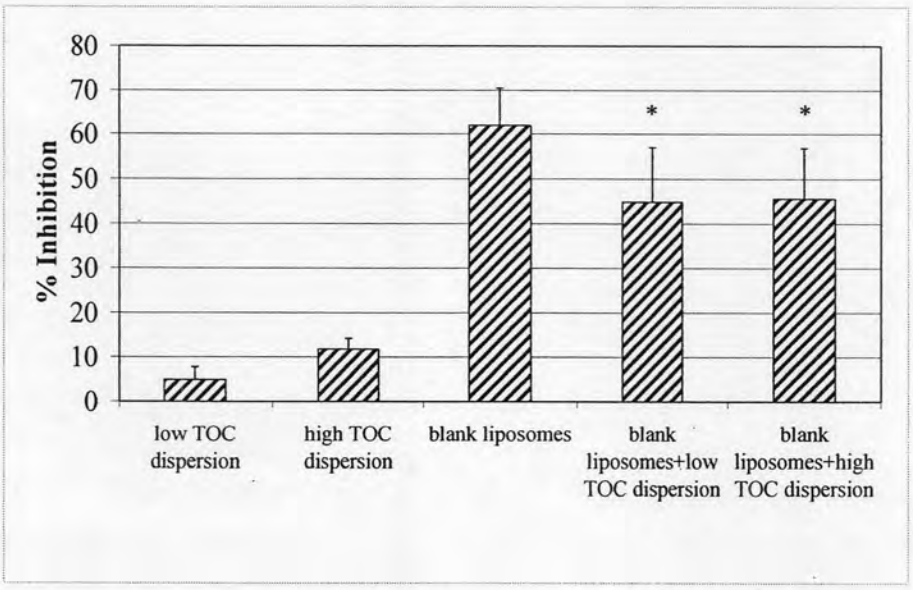

Figure 22: Inhibitory effect of co-incubation of blank PC/DCP liposomes and TOC dispersion on NO production in LPS-stimulated J774A.1 cells. TOC was used at 0.25 mM (low TOC) and 0.5 mM (high TOC). Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. The values displayed are mean ± S.D. (n=6).
* significant difference between liposomes and the corresponding solution ($P<0.05$)

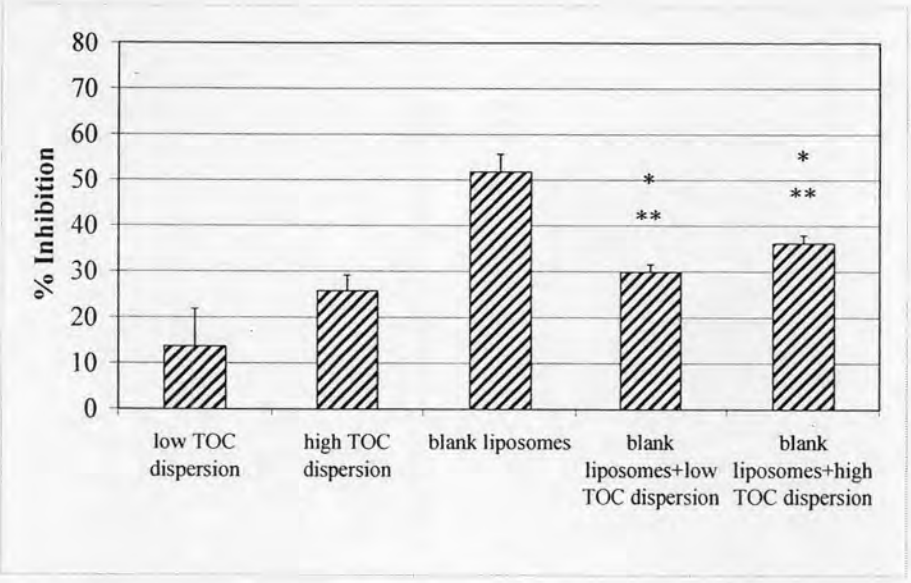

Figure 23: Inhibitory effect of co-incubation of blank PC/PG liposomes and TOC dispersion on NO production in LPS-stimulated J774A.1 cells. TOC was used at 0.25 mM (low TOC) and 0.5 mM (high TOC). Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. The values displayed are mean ± S.D. (n=6).
* significant difference between liposomes and the corresponding solution ($P<0.05$)
** significantly different from blank liposomes ($P<0.05$)

5.2 Effect of co-incubation of blank liposomes and NAC solutions

The inhibitory effect of co-incubation of blank PC/CH liposomes and NAC solution was similar to that seen with NAC-loaded PC/CH liposomes (Figure 24). In contrast to the cases of NAC-loaded negatively charged liposomes, no cytotoxic effect on J774A.1 cells was seen when blank negatively charged liposomes were co-incubated with NAC solutions (Appendices D-9 to D-10). The results from both types of negatively charged liposomes followed the same direction as those of neutral liposomes (Figures 25-26). The overall results were comparable to those of TOC (see Section 5.1 above). However, statistical significance was evident in all cases ($p<0.05$, Appendices C-13 to 15). Though the inhibitory effect of NAC solution on NO production in J774A.1 cells was concentration dependent, increase in NAC concentration did not exert any significant effects when the solution was co-incubated with blank liposomes in all neutral and negatively charged liposomes studied.

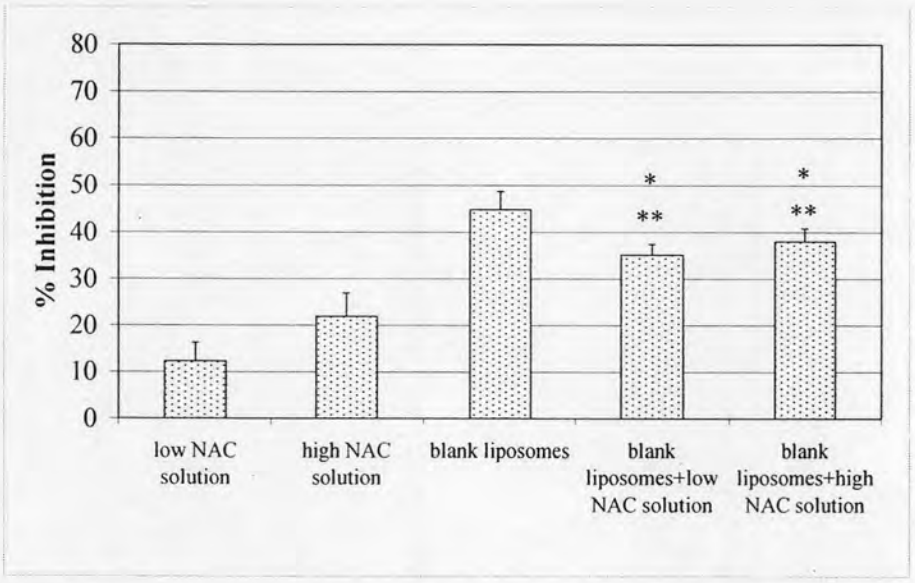

Figure 24: Inhibitory effect of co-incubation of blank PC/CH liposomes and NAC solution on NO production in LPS-stimulated J774A.1 cells. NAC was used at 10 mM (low NAC) and 20 mM (high NAC). Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 µg/ml. The values displayed are mean ± S.D. (n=9).

* significant difference between liposomes and the corresponding solution ($P<0.05$)

** significantly different from blank liposomes ($P<0.05$)

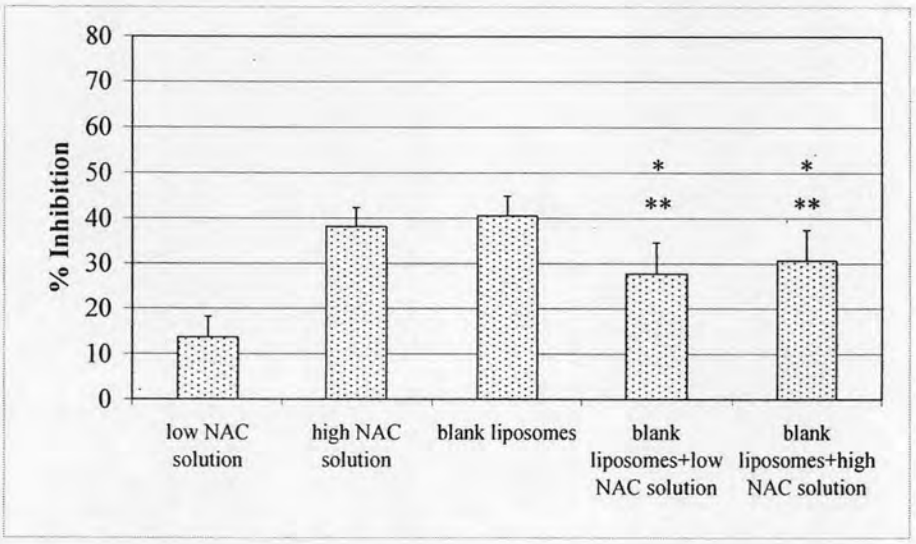

Figure 25: Inhibitory effect of co-incubation of blank PC/CH/DCP liposomes and NAC solution on NO production in LPS-stimulated J774A.1 cells. NAC was used at 10 mM (low NAC) and 20 mM (high NAC). Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 µg/ml. The values displayed are mean ± S.D. (n=9).

* significant difference between liposomes and the corresponding solution ($P<0.05$)

** significantly different from blank liposomes ($P<0.05$)

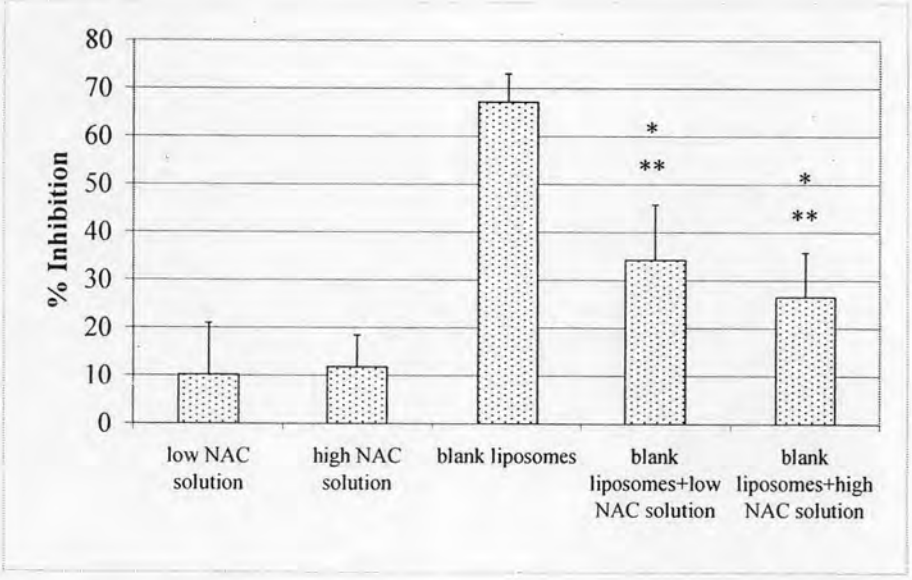

Figure 26: Inhibitory effect of co-incubation of blank PC/CH/PG liposomes and NAC solution on NO production in LPS-stimulated J774A.1 cells. NAC was used at 10 mM (low NAC) and 20 mM (high NAC). Cell seeding density was $1x10^5$cells/ml. LPS concentration was 0.125 μg/ml. The values displayed are mean ± S.D. (n=6).
* significant difference between liposomes and the corresponding solution ($P<0.05$)
** significantly different from blank liposomes ($P<0.05$)

6. Effects of liposome composition on cellular uptake by J774A.1 cells

The aim of this experiment was to compare the extent of cellular uptake between neutral (PC/CH) and negatively charged (PC/CH/DCP) liposomes. Calcein was incorporated into liposomes with as a marker molecule to indicate the degree of uptake of liposomes into the cells.

The percentage of uptake of calcein from solution and from liposome preparations at various time intervals is shown in Figure 27. Calcein uptake from liposomes was increased when compared with the uptake from solution in both types of liposomes at all contact times except at 30 min ($P<0.05$, Appendix C-16). There was no significant difference between calcein uptake from neutral liposomes and negatively charged liposomes during the first 4 hours of incubation. At 24 hours, however, calcein uptake from PC/CH liposomes was significantly higher than that from PC/CH/DCP liposomes ($P<0.05$).

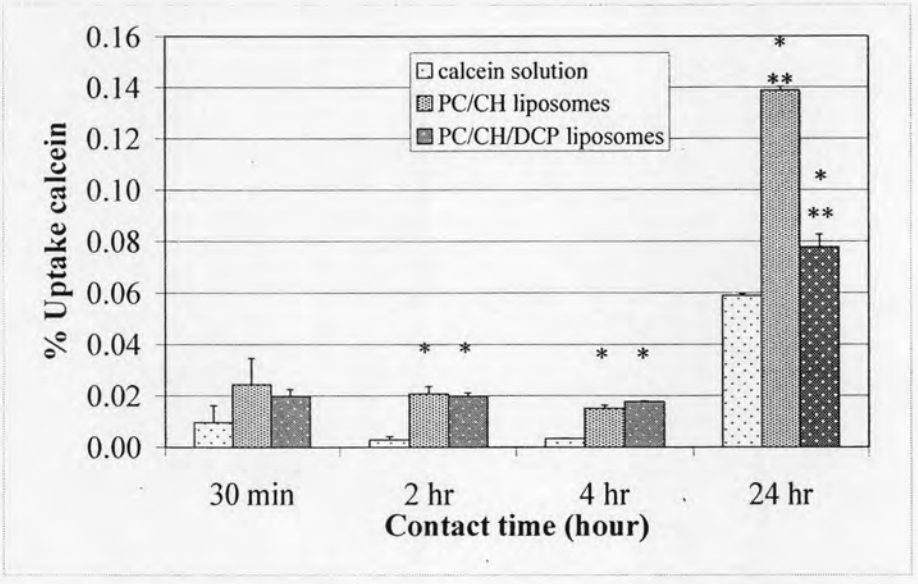

Figure 27: The effect of type of liposomes and contact time on intracellular uptake of calcein in J774A.1 cells. Cell seeding density was $1x10^5$cells/ml. Total lipid concentration was 4 mg/ml. The displayed values are mean ± S.D. (n=3)

* significantly different from solution ($P<0.05$)

** significantly different from each other ($P<0.05$)

7. Effect of NAC-loaded negatively charged liposomes on cell viability

This experiment was designed to determine cytotoxic effect of NAC-loaded PC/CH/DCP liposomes without free NAC at various total lipid concentrations (final conc. = 1-4 mg/ml). As shown in Figure 28, NAC-loaded PC/CH/DCP liposomes without free NAC had only little intrinsic cytotoxic effect on J774A.1 cells at all total lipid concentrations. These results were in contrast with NAC-loaded PC/CH/DCP liposomes in the presence free NAC where serious cytotoxicity took place (see Figures 19-20).

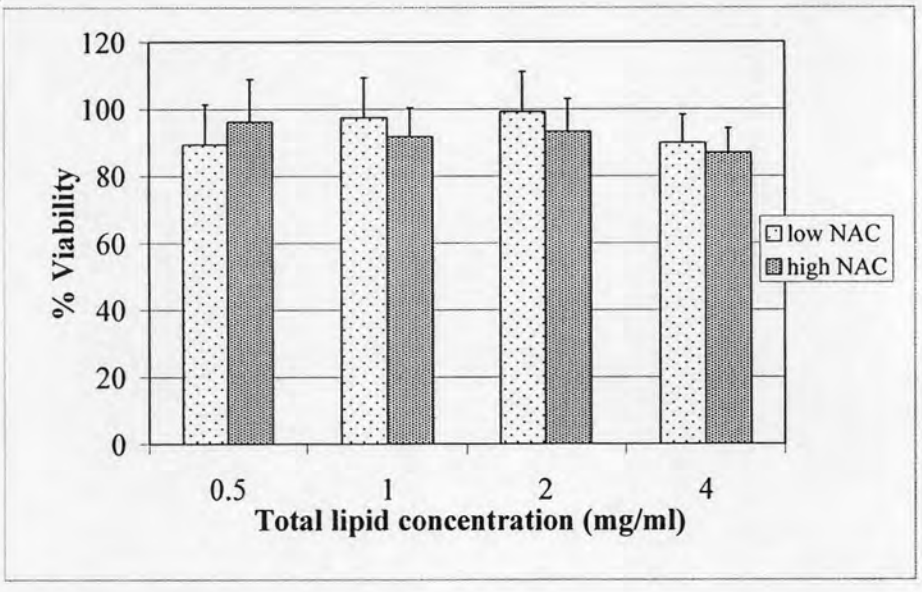

Figure 28: The effect of NAC-loaded PC/CH/DCP liposomes without free NAC on cell viability of J774A.1 cells by MTT assay. NAC was used at 10 mM (low NAC) and 20 mM (high NAC). Cell seeding density was $1x10^5$cells/ml. The displayed values are mean ± S.D. (n=6).

8. Effect of negatively charged liposomes on membrane permeability of a model water-soluble compound

The experiment was designed to investigate whether negatively charged liposomes (PC/CH/DCP liposomes) and NAC-encapsulated negatively charged liposomes (NAC-loaded PC/CH/DCP liposomes) could increase J774A.1 uptake of water-soluble molecules via phagocytosis. The percentage of calcein uptake is shown in Figure 29. The result indicated that NAC-loaded PC/CH/DCP liposomes significantly increased uptake of calcein solution when compared with blank PC/CH/DCP liposomes ($P<0.05$, Appendix C-17). However, there was no significant difference between calcein solution and the other two treatments on intracellular calcein uptake ($P>0.05$).

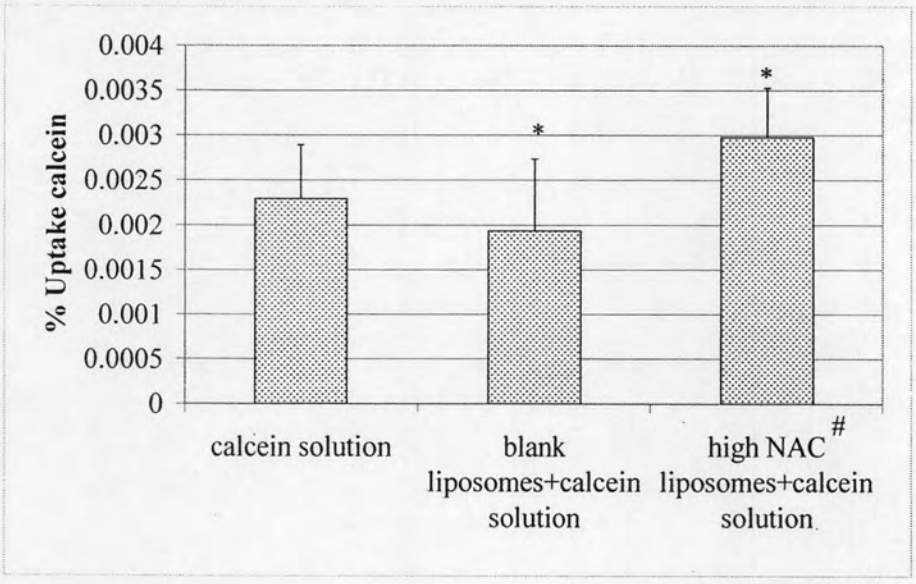

Figure 29: The effect of PC/CH/DCP liposomes on intracellular uptake of calcein solution in J774A.1 cells. Cell seeding density was $1x10^5$cells/ml. Total lipid concentrations was 4 mg/ml and calcein concentration was 0.25 mM. The displayed values are mean ± S.D. (n=6).

NAC-encapsulated PC/CH/DCP liposomes without free NAC

* significantly different from each other ($P<0.05$)