

“Effect of n-Butanol on macrophage functions: An *in vitro* study.”

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Abstract: The modern day lifestyle has witnessed an exposure to different concentrations of n-Butanol in our environment. These compounds are found in different states mostly in dissolved form in aerosols, paints, dyes, medicines and cosmetics etc. However the concentration of environmental exposure in normal conditions is not lethal but it induces a reduced macrophage function in terms phagocytic activity and release of ROS (reactive oxygen species) in response to stress conditions. These alcohols have a delayed degradation time in the body due to higher carbon atoms in comparison to ethanol but the effect on macrophage function is not as pronounced as ethanol at similar concentrations. In this *in vitro* experiment, an exposure of upto 5M n-Butanol proved to be lethal for murine macrophage cells (RAW cell lines) significantly causing cell death. However the effect on phagocytosis and ROS species is pronouncedly less at 2M concentration in comparison to ethanol. Therefore, it indicates at places with high exposure of n-butanol, there is a drastic effect of n-Butanol but in places of normal exposure it leads to a suppressed innate immune response by lowering the phagocytic uptake 20-30% and a slightly reduced release of ROS even at 10^5 cells accumulation.

I. Introduction:

The immune system serves as the body's defense against infections by micro-organisms; damage caused by other foreign substances; and the uncontrolled, tumorous growth of the body's own cells. Impairment of this system can increase a person's risk for developing various illnesses. The immune system has two main arms: **innate**, or non-specific, immunity and **acquired**, or specific, immunity. Innate immunity exists before the body is exposed to a pathogen for the first time. Acquired immunity, in contrast, is activated only after the body is exposed to a pathogen for the first time. The elements of innate immunity include white blood cells that ingest and destroy micro-organisms (i.e., phagocytes); certain proteins that circulate in the blood, called the complement system; and signaling molecules (i.e., cytokines) that are produced and secreted by some of the phagocytes. Several different types of phagocytes exist, with specific functions as follows:

- **Neutrophils** ingest and thereby destroy pathogens, primarily invading bacteria.
- **Monocytes** that circulate in the blood or that have entered the tissues (i.e., macrophages) ingest and destroy a variety of foreign substances and micro-organisms. Monocytes also exhibit pathogen-derived proteins and other molecules (i.e., antigens) on their surfaces in order to activate other cells in the immune system. Finally, monocytes and macrophages secrete cytokines¹ that help regulate immune system activity.
- **Natural killer (NK)** cells recognize and eliminate cells in the body that have been infected by parasites or that have turned into cancer cells.

Alcohol's Effects on Phagocytic cells:

During an inflammatory response, chemical substances released by cells at the site of the infection induce phagocytes to migrate from their normal locations in the bloodstream or the tissues to the site of the inflammation. This process is called chemotaxis. Various substances can serve as chemotactic agents, including activated components of the complement system, a group of white blood cell-derived proteins called leukotriene, and other proteins produced by immune cells (i.e. Chemokine, such as interleukin-8 [IL-8])¹. The Neutrophils and monocytes recruited from the bloodstream must adhere to and migrate through the cell layer lining the blood vessels at the site of the infection, ingest the micro-organisms, and destroy the ingested pathogens using specific enzymes or toxic, oxygen-derived free radicals².

Chronic as well as acute alcohol consumption also reduces the ability of phagocytes to ingest and break down pathogenic bacteria. For example, cultured human monocytes exposed to alcohol showed reduced phagocytic functions; moreover, the cells produced less of a receptor protein that is required for the ingestion of antibody-coated particles. In mice, both short-term and long-term alcohol feeding reduced the phagocytic ability of macrophages residing in the membrane lining the abdominal cavity². Thus, abnormal neutrophil adherence and chemotaxis, as well as reduced phagocytic function of macrophages, may contribute to the impaired defense against micro-organisms observed after alcohol consumption.

Alcohol's Effects on Oxygen-Radical Production:

Oxygen radicals (e.g., **super oxide anions and hydrogen peroxide**) are unstable oxygen-containing molecules that readily interact with other molecules in a cell. Oxygen radicals produced by macrophages and

other phagocytes play a crucial role in destroying micro-organisms, especially in the lungs. Researchers found that macrophages in the lungs of acutely or chronically alcohol-fed rats produced fewer super-oxide anions and less hydrogen peroxide than did macrophages from non-alcohol exposed rats². Furthermore, the lung macrophages produced and secreted less **nitric oxide**, another molecule with characteristics and functions similar to those of oxygen radicals³. The alcohol induced decreases in the macrophages' production of oxygen radicals and nitric oxide could undermine the body's defense against bacteria². This mechanism could contribute to the high incidence of tuberculosis in alcoholics.

IN VITRO CULTURE AND MAINTENANCE OF RAW 264.7 CELL Lines: an adherent murine macrophage cell line

Adherent Cell Line: - The adherent cell lines are typically fibroblast (connective tissue) vascular endothelial cells or smooth muscle cells with elongated, spindle shaped morphology. The adhesion of cells to the surface of the plastic dish is caused by extra cellular protein (adhesion molecules). Cell adhesion is mediated by specific cell surface receptor molecules in the extra-cellular matrix, so it seems likely that cell spreading may be preceded by secretion of extra-cellular matrix proteins and proteoglycans by the cells. Three major classes of trans-membrane proteins have been shown to be involved in cell-cell and cell substrate adhesion. Cell-cell adhesion molecules, CAMs and Catherins involved primarily in interaction between homogenous cells. They are self-interactive, i.e. like molecules in opposing cells interact with each other. Cell substrate interactions are mediated primarily by integrin, receptor for matrix molecules such as fibronectin, laminin, and collagen. The third group of cell adhesion molecules is the transmembrane proteoglycans also interacting with matrix constituents such as other proteoglycans or collagen.

1-Butanol is used primarily as a chemical intermediate in the production of butyl acrylate and methacrylate. It is also used in the production of glycol ethers and butyl acetate; as a solvent; and as a plasticizer (Mannsville 1993). Small amounts are also used in hydraulic fluids, in detergent formulations, as a medication, and as an extractant in the manufacture of pharmaceuticals. 1-Butanol are released to the environment from natural and human sources. Other natural sources include animal wastes, microbes and insects. Human sources of 1-butanol include volatilization from solvents, rendering, sewage treatment, starch manufacture, whiskey manufacture, wood pulping and turbine emissions. 1-Butanol may be released to water through various wastewater emissions, including those from chemical manufacturing plants, textile plants, pulp mills making kraft paper, sewage treatment plants, oil refineries, and landfill leachates. The effluents from a petrochemical facility contained 16.0 mg/L 1-butanol; the daily discharge was ~90 pounds/day.

HEALTH EFFECTS

A. Pharmacokinetics

Absorption - Studies in humans and animals have demonstrated that 1-butanol is readily absorbed by the respiratory tract, the gastrointestinal tract, and the skin. The total absorption by humans exposed by inhalation and skin is of concern for workers. In vivo and in vitro studies have shown that the chemical is also absorbed through the oral mucosa of dogs and the cornea of rabbits' eyes.

B. Acute Effects

Acute inhalation exposure to low to moderate levels of 1-butanol produces irritation of the eyes, nose and throat in humans. Exposure to high, non-lethal levels cause irritation in animals³. Exposure to high, non-lethal levels for several days causes reversible liver and kidney effects in animals.

C. Chronic Effects:

Humans exposed chronically to high concentrations of 1-butanol have experienced neurological, ocular, auditory, and dermal effects. Animals exposed chronically to 1-butanol exhibit vascular changes, increased thyroid activity, and pathological lesions of the lungs and intestines at low to moderate concentrations, and degenerative changes of the liver and kidneys, and effects on the blood³.

D. Carcinogenicity

No information was found for the carcinogenicity of 1-butanol in humans or animals.

G. Neurotoxicity

1-Butanol affects the human central nervous system, producing adverse effects at moderate concentrations in air. 1-Butanol causes adverse central nervous system effects in animals by both the oral and inhalation routes of exposure.

Further the effect of the alcohol is checked by its effect on Macrophage function, by focusing on two parameters-

1. Phagocytic Uptake
2. NO Release (Griess Reagent).

To check the phagocytic uptake efficiency of macrophage cells **Crystal Violet stain** is performed at 540nm and NO release is being estimated with **Griess Reagent**. The experiment is also repeated simultaneously with, LPS activated Macrophages. In general, the activation of macrophages by addition of LPS increases the phagocytic uptake and also NO in response to a particular concentration of alcohol in comparison the inactivated macrophages⁴. Lipopolysaccharide (LPS) stimulates immune responses by interacting with the membrane receptor CD14 to induce the generation of cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, and IL-6^{8,12,10}.

Methyl violet 10B has six methyl groups. It is known in medicine as Gentian violet (or crystal violet or pyocyanine and is the active ingredient in a Gram stain, used to classify bacteria. It is used as a pH indicator, with a range between 0 and 1.6. Methyl violet 10B also binds to DNA. This means it can be used in cell viability assays in biochemistry.

The **Griess test** is a chemical analysis test which detects the presence of organic nitrite compounds. Nitrite is detected and analyzed by formation of a red pink colour upon treatment of a NO₂⁻-containing sample with the Griess reagent. When sulphanilic acid is added, the nitrites form a diazonium salt. When the azo dye agent (alpha-naphthylamine) is added a pink colour develops.

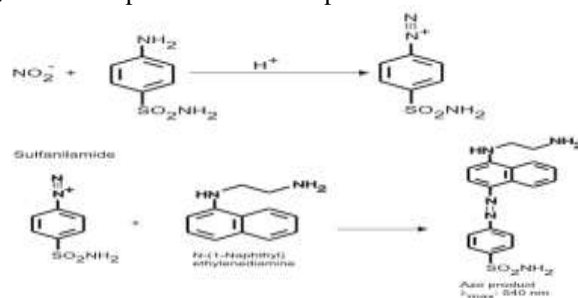


Fig.1 Reaction of components of Griess reagent.

II. Methods:

RAW cells culture:

RAW 264.7 Cells are maintained at 37 °C in a humidified atmosphere with 5% CO₂. For routine maintenance in culture (passage), cells are seeded at a confluence of approximately 10% (1 x 10⁶ and 3 x 10⁶ cells in 100-mm and 150-mm plates, respectively) and grown to a confluence of approximately 80%. This procedure requires the cells to be split every two days. Cultures are not maintained beyond three months. New cultures are thawed at least two weeks prior to end of the three-month culture period. Murine macrophages and macrophage-like cell lines such as RAW 264.7 adhere to tissue culture-grade plastic through cation-dependent integrin receptors and other cation-independent receptors, predominantly the murine scavenger receptors (MSRs). In order to reduce adhesion during routine culture, the RAW 264.7 cells are grown on sterile non-tissue-grade plastic (ultra-dish Petri dishes). Adherence of macrophages to these plates is mediated by $\alpha\text{M}\beta 2$ (CR3) integrin and this interaction is readily reversed by using cation chelators such as EDTA. This adhesive interaction is also sufficiently weak that cells may be detached by the sheer force of media flowing over the cells.

CELL STORAGE AND CELL REVIVAL: -Cell lines show age related changes *in vitro* and some cultures may undergo spontaneous transformation to exhibit cultured growth or changes in functional characteristics cells that are properly frozen can be kept for extended period. In order to survive freezing thawing, cells must be treated with a cry protective agent. Dimethyl sulfoxide (DMSO) is added to the suspension medium acts to permeabilize the plasma membrane and allows water to flow out of the cell as cooling occurs cryoprotectants depress the freezing point so that ice crystals begin to form at about -5°C.

Treatment of RAW cells with *n*-butanol:

The study involved the treatment of cultured RAW cells to different concentration of alcohol. This required the standardization of the number of cells to be plated for treatment, the concentration of alcohol to be used and the time duration. The following experiments have been carried out for these purposes.

Standardization of Number of cells for treatment:

This experiment was done with the main objective of determining the number of cells that are required to be plated so that phagocytosis can be seen easily and there is no overcrowding of cells. For this, we took a single 4-well plate and plated four different numbers of cells:

1. 10^4 cells
2. 2×10^4 cells
3. 5×10^4 cells
4. 10^3 cells

The total concentration of cells as counted in a Neubauer chamber was 8.83×10^5 cells. Thus the amounts of suspension to be plated in 200 microliter (μ l) medium in each well can be calculated as:

Conc. of cells to be plated X Volume of sample

Conc. of cells in suspension sample

Thus, the volume of suspension in each well will be 11 μ l, 22 μ l and 55 μ l respectively. For 10^3 cells, 10 times dilution is done and then 11 μ l is plated. The medium is plated accordingly. The culture is kept at 37°C in 5% carbon dioxide incubator for 24 hours. Phagocytosis assay is done by using yeast.

Standardization of Concentration for treatment:

Different doses of **25mM, 100mM, 1M, 2M, 5M** were prepared to be taken keeping in consideration the v/v % which is in the range of **0.1-0.5**. This is important because several drugs using alcohol have a v/v% of **0.01** which is healthy for the cells. In single well plates the concentration of cells plated was 10^4 cells/ml and the amount of media taken was 1ml. The plating was done as follows:

1. Control plate

Three for each of the following treatment

- A. 25mM
- B. 100mM
- C. 1M
- D. 2 M
- E. 5M

The cells were treated with respective concentration of the alcohol for specified time period (**24 hrs. 48 hrs.**) and then incubated at 37°C in 5% carbon dioxide incubator for 24 hours. The cells were checked for viability by crystal violet assay of cells after fixed time duration of treatment and the absorbance at 540nm was noted down.

Treatment of LPS-activated Macrophages to different concentrations of ethanol:

This experiment was conducted to determine the change in phagocytic activity of macrophages on treatment with different concentration of ethanol followed by their activation by adding LPS. For this, the same process was repeated as in case of inactivated macrophages, except that 2 μ l LPS was added to the wells to study activation, after addition of the treatment. The phagocytic assay was done the next day to determine the change in activity same as that of inactivated macrophages.

The protocol for phagocytic assay is as follows:

Yeast suspension (1mg/ml) was made in an eppendorf tube. 200 μ l yeast suspension was added in respective wells and incubated for 5min in water bath maintained at 37°C. Culture medium was carefully removed from wells. Plates were gently with warm PBS (500 μ l) warmed to room temperature three times. Then the PBS was removed and 200 μ l methanol was added to fix the cells and allowed it to dry after removing methanol. 200 μ l Crystal violet was added and incubated for 1min at room temperature. The plate was washed twice in tap water. Water can be removed by simple flicking of plate. The cells were observed under the microscope and phagocytic activity was determined.

Nitric Oxide Assay:

NO production was estimated by measuring the amount of nitrite in the culture supernatant using Griess reagent. In this, an aliquot of the conditioned medium was mixed with an equal volume of 1% Sulphanilamide and 0.1% N-1-naphthylethylene-diamine dihydrochloride in 5% phosphoric acid. The absorbance at 540nm was measured to evaluate NO production with an ELISA plate reader.

III. Results And Discussion:

Standardization of Number of cells for Treatment: On the basis of the graph, we conclude that 2×10^4 cells is the optimum number as it gives us a scope to determine changes due to n-butanol treatment and also there is no overcrowding as in 50,000 cells.

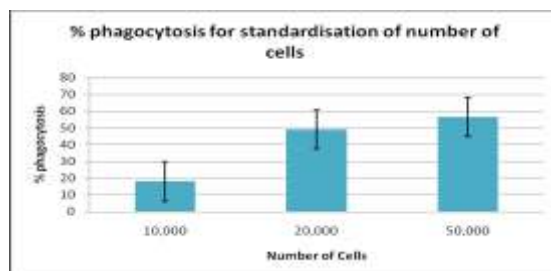


Fig.2. Standardisation of number of Cells for treatment.

10^3 cells were too less to conduct phagocytic assay. As the macrophages are dark stained it is important to have sufficient resolution in scoring the phagocytosis of each macrophage cell. Moreover the plate size on which the experiment is to be performed also limits the cell number to be used in the experiment and is detrimental to have an error free count.

The following figures show phagocytosis in different wells containing different number of cells. The macrophages are seen as blue and yeast as smaller particles with a darker outline attached to the macrophages.

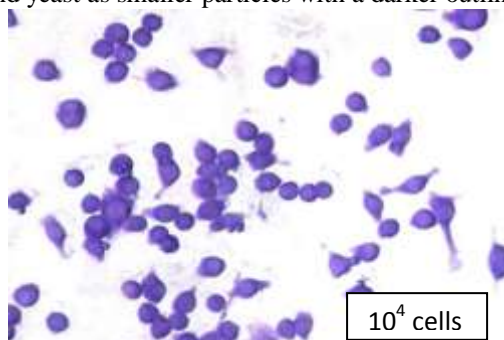


Fig.3. Macrophage cells stained with crystal violet viewed under microscope.

Cell Viability Check:

Cell viability check was done with crystal violet reagent at 540nm. In this assay O.D was taken at 24 and 48 hrs. time period.

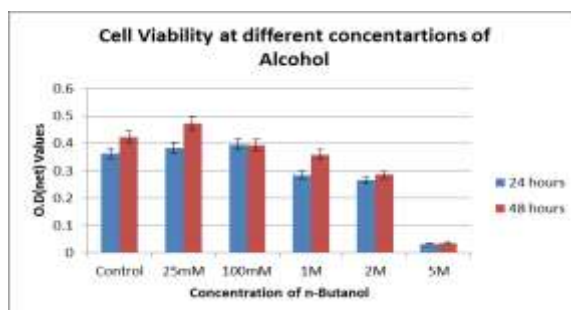


Fig.3. Cell viability check at 540nm of light at 24 and 48 hours.

The values of O.D (net) indicate that cells were not viable enough at 5M for in vitro experimental conditions. Most of the cells were dead, which was confirmed using **Trypan Blue assay**. This suggested that in vitro study of effect of n-Butanol is limited to a concentration of 5M. So, concentrations below this are used for analysis. At 5M cell ceases to proliferate and most cells are dead. However in comparison to common used alcohol, ethanol, in-vitro studies have been seen to be lethal at 2M concentration³. This indicates that the exposure of n-butanol in our day to day life is not lethal, but the cell health has prominent effect leading to a decline in cell proliferation.

Treatment of macrophage cells with different concentration of alcohol:

The macrophages show an increased activation on addition of LPS.

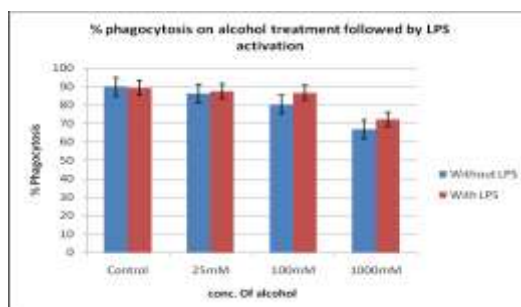


Fig.4. Phagocytosis percentage followed by LPS activation.

Under the effect of n-Butanol at increasing concentrations of alcohol the phagocytosis efficiency decrease however this effect is not so prominent in terms of percent phagocytosis. Also, LPS- activated macrophages show a decrease in phagocytosis on exposure to different concentrations of alcohol. This may imply that Alcohol causes injury to macrophages. However upto 100mM concentration of alcohol activation by LPS is not so influential. A significant change in phagocytic uptake is noticed after 100mM concentration. This indicates that the macrophage cells require higher concentration of n-butanol to show effect. On comparing with the effect of ethanol on macrophage cells this trend is quite more appreciable, as there is a rapid hike in phagocytosis of yeast after 25mM concentration.

In order to make this study more accurate the phagocytic index was calculated, taking into consideration the effective number of yeast cells up taken and the percent phagocytosis.

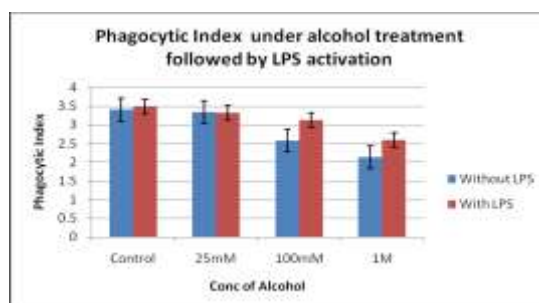


Fig.5 Phagocytic Index followed by LPS activation.

These pictures show increased activation and altered morphology of macrophages on treatment with LPS. Activation of macrophages by bacterial lipopolysaccharide (LPS) induces transcription of genes that encode for proinflammatory regulators of the immune response. On Activation, macrophages undergo a number of morphological, biochemical and functional alteration like increase in adhesion, spreading, ruffling of plasma membrane etc.

Nitric Oxide assay to determine nitric oxide release:

Result after 24 hours was seen as an insignificant rise in NO release with increasing concentration of alcohol. This was most prominent at a concentration of 1M, which showed the effect of n-Butanol on the macrophage function. This substantiated the fact the release of ROS, a vital part in immunity elicitation is drastically hampered at increasing concentrations of n-Butanol. However if compared to NO release by LPS activated macrophages it is significant at lower concentrations. But at 1M LPS activation of macrophages has no significant ROS release.

The graphical representation is as follows:

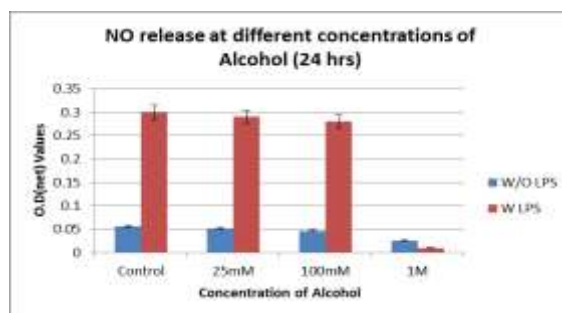


Fig.6.NO release followed by LPS activation after 24 hours.

To study the prolonged effect of LPS activation of macrophages on ROS release, the OD values were taken after 48 hours. Result after 48 hours also depicted a decrease in NO release with increasing concentration of Alcohol. But as of 24 hours exposure, here in this case there was a significant release of ROS at 1M concentration. This indicates the fact that increases in number of cell also aids release of ROS species².

The graphical representation is as follows:

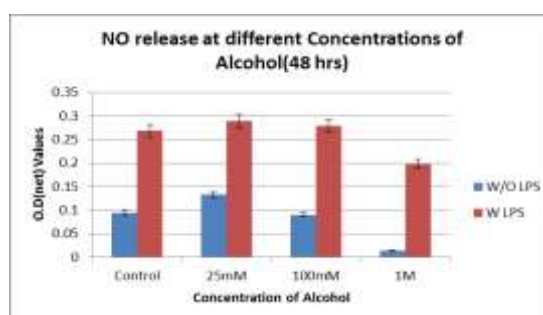


Fig.7.NO release followed by LPS activation after 48 hours.

The release of NO and the uptake of yeast cells however stand different in regards to the higher alcohol concentrations. This suggests that the degradation of a four carbon chain alcohol with time varies and thus influences different components of immune system differently.

IV. Conclusion:

Alcohol (n-butanol) certainly has a profound effect on the function and morphology of macrophages. Alcohol (5M) in invitro conditions has a lethal effect on the cells.n-Butanol at viable concentrations does not cause direct effect but show modulation in function of macrophages. In this study, it can be concluded that 2M alcohol concentration has the most profound effect on phagocytic activity of macrophages and nitric oxide release. There is a decrease in the phagocytic activity of macrophages with increasing concentration of alcohol and cell dies almost around 5M alcohol concentrations in invitro conditions. In present study, it was also found that phagocytic activity of LPS-activated macrophages also decreased with increasing concentration of alcohol. However, the nitric oxide released on activation of macrophages by lipopolysaccharide shows an increasing trend implying that alcohol causes some injury and thus an increase in release of nitric oxide². Therefore alcohol has a dual effect on immune system. Firstly, it suppresses the immune system by decreasing phagocytic activity of macrophages. Secondly, it is inducing inflammatory conditions by increasing Nitric oxide release by macrophages⁵. This also supports the fact that phagocytosis and nitric oxide release follow different mechanisms.

Alcoholics frequently suffer from infectious diseases and have increased rates of some cancers, indicating that alcohol impairs the immune system, which protects the body against this type of damage^{3, 4}. Alcohol interferes with the functions of many of the cells and molecules that are part of the immune system. For example, alcohol inhibits the functions of the cells that ingest and destroy invading micro-organisms³ (i.e., neutrophils, monocytes, and macrophages). Both acute and chronic alcohol exposure also alter the production of signaling molecules that help coordinate the immune response (i.e., cytokines)¹⁰.

FUTURE PROSPECTS:

Since we have defined the change in function of two parameters including nitric oxide release, we can determine the effect of alcohol also by **cytokine analysis** and reactive oxygen species (**ROS**) analysis. We can also determine the effect of alcohol at the genetic level by checking the expression of iNOS gene. Also, the expression can also be determined by quantifying protein by western blotting and analysing the correlation obtained. These results can help us in understanding how the macrophages function against alcohol, their role in development of cancer and most importantly for finding a cure against cancer. Further investigation can be done on the mechanisms underlying the differential effects of chronic and acute alcohol use on the immune system.

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