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In vitro effect of tuibur (tobacco brew) on the viability of human blood lymphocytes

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The use of tobacco and its products are known to cause many illnesses including cancer. A smokeless tobacco locally manufactured called *tuibur* (tobacco brew) has been consumed by the Mizos from a very long time. In this experiment we aim to determine the cytotoxicity of *tuibur* by an *in vitro* study on *tuibur*-treated human peripheral blood lymphocytes. We have found that 24 h treatment of human lymphocytes with two grades of commercial *tuibur* and nicotine showed a concentration dependent decrease in cell viability. We, therefore, concluded that as the *in vitro* use of *tuibur* has an adverse effect on cell survival, its consumption might have potential side effects on the health of the users.

Key words: Cell viability, lymphocytes, tobacco, tuibur.

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Introduction

Tobacco is linked with many diseases and has been known to contain more than eight thousand chemicals, out of which roughly 68 are probable carcinogens.[1-3] Some of the common toxic chemicals include benzo[a]pyrene (B[a]P), N′-nitrosonornicotine (NNN), N′-nitrosoanatabine (NAT), N′-nitrosoanabasine (NAB), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosodimethylamine (NDMA), nitrite, cadmium, lead, arsenic, nickel, chromium, etc.[4,5] The consumption of both smoking and smokeless tobacco is popular throughout the world and its detrimental effect could be observed from many medical records. Besides its deleterious consequence upon the pulmonary system, it has been linked with many forms of cancer. In fact, many studies suggested that almost all known cancer could be linked to tobacco use.[6,7]

It would be safe to say that every nation throughout the globe has tobacco users in its population.[8] The form of tobacco used may vary considerably. Some prefer smoking tobacco while others prefer smokeless tobacco, or both. But, it may be acceptable to say that more than half of the tobacco users used it in the form of smoking tobacco.[9] The Mizo tribes living in the northeastern part of India use both smoke and smokeless tobacco.[10] A form of smokeless tobacco locally called *tuibur* (tobacco brew) is used popularly and is commercially available in the

local market, generally in two grades, which largely depend on the amount of tobacco used in its production. The method of practice is the users of *tuibur* put the product in the mouth for roughly 5-10 minutes which is then spitted out. The duration is determined when the alkalinity of the *tuibur* is depleted.[11]

In this experiment, we aimed to determine the effect of two grades of commercial *tuibur* on the viability of *tuibur*-treated human peripheral blood lymphocytes *in vitro*.

Materials and Methods

Chemicals

A small quantity of two grades of commercial *tuibur*, labelled as *tuibur*-A (special grade) and *tuibur*-B (ordinary grade), produced in a local industry were purchased from the market. Although there is no standard protocol, the manufacturers graded the *tuibur* depending on the quantity of tobacco used in its production. Pure nicotine (Cayman Chemical Company) and trypan blue (Sigma) were purchased from local supplier. RPMI-1640 media (HiMedia) was obtained from local supplier and prepared in the laboratory using standard protocol.

Lymphocyte culture and treatment

Lymphocyte culture were performed using the protocol described by Jagetia *et al.* [12] Briefly, peripheral blood lymphocytes were collected by venipuncture in a heparinized vacutainer from a 27-year-old healthy male volunteer who has no known history of tobacco consumption. The collected blood was allowed to stand for roughly half an hour and the upper translucent layer containing lymphocytes was taken for culture. Approximately two million lymphocytes were cultured in different test tubes containing 2 ml RPMI-1640 culture media without the addition of any growth factor.

The tubes were separated into four groups (I, II, III & IV) and different volumes of *tuibur*-A and *tuibur*-B were added to group I & II (2.5, 5, 10, 20, 40, and 50 µl/ml) respectively. To group III, 2.5, 10, 20, 40, and 50 µg/ml of nicotine was added and this served as positive control. Group IV or blank acted as negative control and did not contain any chemical other than the cells and the media. All cultures were performed in triplicate. These tubes were incubated at 37°C for 24 h. After 24 h, the survival of the cells was checked by modified trypan blue exclusion assay.[13] The number of living and dead cells were counted in a hemocytometer and the mean percentage of surviving cells was taken as viability.

Statistical analysis

All statistical analysis were performed using Microsoft Excel 2013 and OriginPro-8. Correla-

Table 1 | Mean percentage of viable human peripheral blood lymphocytes for blank and treatment with different concentration of *tuibur*-A, *tuibur*-B and nicotine.

Concentration (µl/ml or µg/ml)	Mean % of viable cells±SEM			
	Tuibur-A	*Tuibur*-B	Nicotine	Blank
0	-	-	-	100.00±0.00
2.5	100.00±0.00	100.00±0.00	98.25±0.06	-
5	96.39±0.58	95.06±0.40	96.55±0.25	-
10	90.34±1.86	90.35±0.97	92.32±0.59	-
20	86.41±0.62	87.03±0.29	91.1±0.23	-
30	79.43±2.22	85.65±1.20	87.9±0.50	-
40	71.07±1.97	79.15±0.58	85.57±0.14	-
50	65.57±0.62	76.14±1.11	82.71±2.31	-

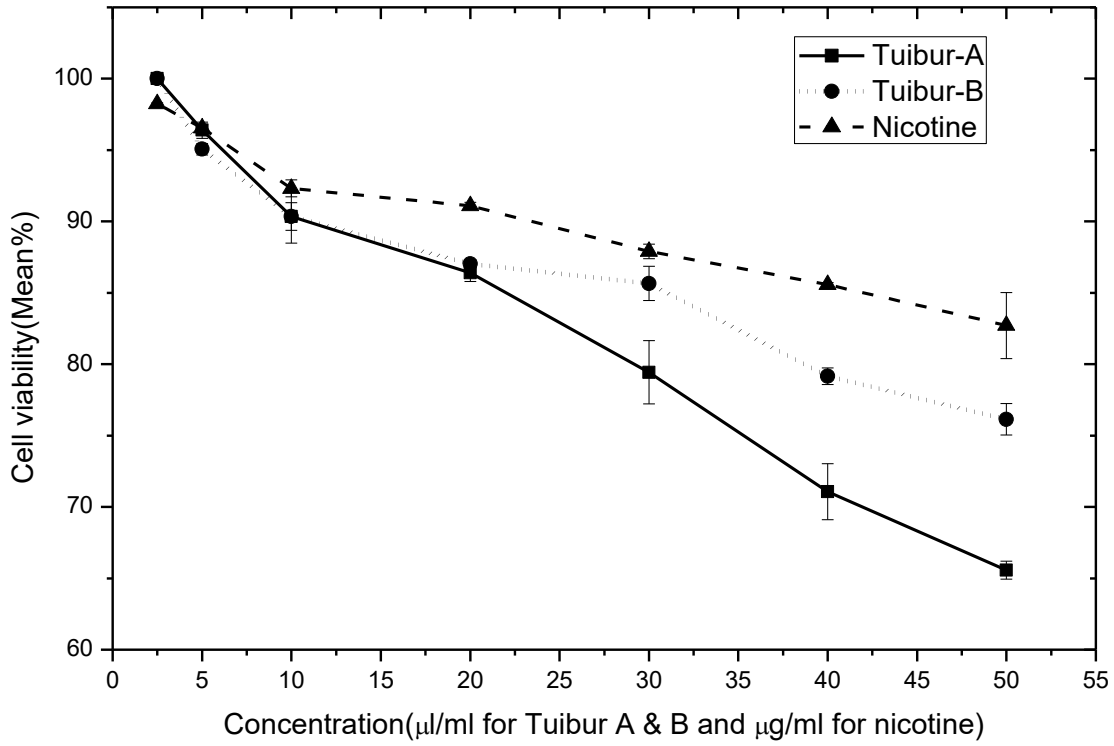

Figure 1 | Graph showing mean percentage of viable human peripheral blood lymphocytes treated with different concentration of *tuibur*-A, *tuibur*-B and nicotine.

tion coefficient was performed to determine relationship between different treatment concentrations and viability within a group. Student's *t*-test was employed to determine significant difference between the treatment groups.

Table 2 | Student's *t*-test between different treatment groups at 95% confidence interval.

Student's t-test between	p-value at 95% CI	Inference
Tuibur-A & *Tuibur*-B	0.60	No significant difference
Tuibur-A & Nicotine	0.27	No significant difference
Tuibur-B & Nicotine	0.45	No significant difference
Control & *Tuibur*-A	≤0.00	Significant difference
Control & *Tuibur*-B	≤0.00	Significant difference
Control & Nicotine	≤0.00	Significant difference

Results

The pH of *tuibur*-A and *tuibur*-B were found to be 9.81 and 10.09 respectively. Table 1 and Figure 1 showed the mean percentage of viable cells for the different treatment groups. The negative control showed 100% viability while *tuibur*-A, *tuibur*-B and nicotine showed a concentration dependent viability. Lymphocytes treated with a maximum concentration of 50 µl/mlof *tuibur*-A and *tuibur*-B showed 65.57% and 76.14% viability respectively while a minimum concentration of 2.5 µl/ml of both the two *tuibur* grades resulted in 100% viability in both the groups. A maximum concentration of 50 µg/ml and a minimum concentration of 2.5 µg/ml of nicotine showed 82.71% and 98.25% viability respectively. A strong negative correlation was observed between cell viability and concentration of *tuibur*-A (-0.994), *tuibur*-B (-0.969) and

nicotine (-0.979). This means higher the concentration of the chemicals, lower the viability and vice versa.

Statistical analysis by t-test at 95% CI (Table 2) between mean percentage of viable cells for blank and *tuibur*-A, blank and *tuibur*-B, blank and nicotine showed a significant difference (p-value≤0.00). However, comparison of *tuibur*-A and *tuibur*-B (p-value=0.60), *tuibur*-A and nicotine (p-value=0.27), *tuibur*-B and nicotine (p-value=0.45) showed that there is no significant difference in mean percentage of viable cells between these groups.

Discussion

Tobacco is known to contain enormous amount of different chemicals, many of which have been reported to have carcinogenic and cytotoxic properties.[1,14] Most studies, if not all, reported the use of tobacco in any form only have negative impact on the physiological well-being of the users. There have been only a handful of literatures on the scientific investigation of *tuibur*. A preliminary report on the chemical composition of *tuibur* showed the presence of polyaromatic hydrocarbons and carbonyl compounds in the tar phase.[11]

An epidemiological study among the Mizos showed that *tuibur* users have a higher risk of developing gastric cancer and the combine use and frequency of smoking, betel, *tuibur* and *sahdah* were reported to have a significant influence on the risk of gastric cancer.[10] Phukan *et al.*[15] have also reported *tuibur* use as a risk factor for gastric cancer. Besides gastric cancer patients in Mizoram, *tuibur* consumers were found to have a variety of mtDNA D-loop region mutations and polymorphisms.[16] Individuals with Arg/Pro genotype,GSTM1 null genotype and GSTT1 non-null genotype were also suggested to have a higher risk of gastric cancer if they have habits of using *tuibur* and smoking tobacco.[17,18]

The damaging effect of tobacco may be attributed to its vast array of chemical compositions. Heavy metals like cadmium and lead present in tobacco have also been found to cause glomerular dysfunction. Many of these effects may be because of nicotine's ability to affect certain antioxidant enzymes like lipid peroxidase, superoxide dismutase, catalase, glutathione-s-transferase, glutathione reductase, etc.[3] Cytological studies have reported nicotine to inhibited cell proliferation and decreased protein synthesis in a dose dependent manner in cultured periodontal ligament fibroblast,[19] while it was also reported to stimulate endothelial cell DNA synthesis and proliferation at concentrations lower than $<10^{-8}$ M. The cytotoxicity of nicotine was reported to be at a higher concentration, i.e. $>10^{-6}$.[20]

Onion bulbs treated with *tuibur* showed a reduced root growth, reduced mitotic index, formation of micronuclei, lagging chromosomes, and c-mitosis.[21] A study on seven smokeless tobacco aqueous extracts showed a concentration-dependent effects on the growth and viability of oral bacteria cultured under anaerobic conditions.[22] These effects may be a result of increase superoxide anion production, lipid peroxidation, DNA fragmentation and DNA ladders caused by the use of chewing tobaccos.[23]

Our result showed concentration dependent cell viability for the *tuibur* and nicotine treatment groups while the untreated negative control group showed 100% viability. We are uncertain as to what chemical(s) in the tobacco brew would cause the cells to die. But from the nicotine treatment group, we may be able to say, although carefully, that the nicotine might contributed significantly in this result. However, one study suggested other biologically active compounds like NNN, NNK, etc., other than nicotine present in tobacco leave extract to be the source of cytotoxicity.[24]

Another probable factor for the decrease in viability of the *tuibur* treatment groups would be the change in pH of the culture media. As we have shown in our result, the pH of both the two grades of *tuibur* are alkaline in nature, a slight rise in pH of the culture media was observed after the addition of both the *tuibur* (data not shown). This change in pH may be a factor that leads to decrease cell viability. In conclusion, our

result showed that 24 h treatment of human lymphocytes with *tuibur* and nicotine may have an adverse effect on their survival and hence these chemicals might have cytotoxic properties. Therefore, the consumption of *tuibur* might have potential side effects on the health of the users.

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