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Brief Report

A Simple Way to Reduce the Risk of Cancer of the Oral Cavity, Pharynx, Larynx and Esophagus in Alcohol Users

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Abstract: Almost 6% of cancers worldwide are attributable to alcohol consumption. Approximately half of them occur in tissues highly exposed to ethanol, such as the oral cavity, pharynx, upper larynx and esophagus. However, since ethanol is not mutagenic and the carcinogenic metabolite of ethanol (acetaldehyde) is mainly produced in the liver, it is unclear why alcohol consumption preferentially causes a local carcinogenic effect. We recently hypothesized that the cytotoxic activity of ethanol could explain the high risk of these cancers in alcohol users. Here we report that short-term exposures (2-3 seconds) to ethanol concentrations between 10% and 15% start to cause a marked cytotoxic effect on human epithelial keratinocytes in a concentration-dependent manner. After discussing new evidence that cancer is the end-result of the accumulation of cell divisions in stem cells, we explain why regular alcohol consumption imposes a high risk of cancer on these tissues. Briefly, the cytotoxicity of ethanol reduces the lifespan of the cells lining these tissues. The stem cells located in deeper layers need to divide more often than usual to renew the damaged epithelia. The accumulation of cell divisions in stem cells leads to the accumulation of cancer-promoting errors (e.g., mutations arising during DNA replication) that increase their risk of malignant transformation. Cell division also exposes the DNA of the stem cells to the genotoxic activity of acetaldehyde and tobacco carcinogens. We propose that choosing alcoholic beverages containing non-cytotoxic concentrations of ethanol, or diluting ethanol to non-cytotoxic concentrations, is a simple way to reduce the risk of cancer of the oral cavity, pharynx, larynx and esophagus in alcohol users. This preventive strategy may also abolish the known synergistic effect of alcohol drinking and tobacco smoking on the risk of these cancers.

Keywords: alcohol consumption; tobacco smoking; carcinogenesis; addiction; stem cells; stem cell division theory of cancer

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Introduction

Alcohol consumption is carcinogenic to humans. According to the International Agency for Research on Cancer (IARC), alcohol consumption causes cancer of the oral cavity, pharynx, larynx, esophagus, colorectum, liver (hepatocellular carcinoma) and female breast [1]. The risk is particularly high for tissues directly exposed to ethanol. For example, compared with non-drinkers, the relative risk for heavy alcohol users is 5.13 for oropharyngeal cancer, 4.95 for esophageal cancer, 2.65 for laryngeal cancer, 2.07 for liver cancer, 1.44 for colorectal cancer and 1.61 for breast cancer [2]. It has been estimated that almost 6% of the total number of cancer cases and deaths world-wide are attributable to alcohol consumption, and that approximately half of them occur in tissues highly exposed to ethanol, such as the oral cavity, pharynx, larynx and esophagus [3].

The mechanism by which alcohol consumption preferentially exerts a local carcinogenic effect remains unclear [1,4]. Since ethanol is not mutagenic, it is widely accepted that the carcinogenic activity of alcohol consumption is mediated by acetaldehyde, a mutagenic metabolite of ethanol. Upon ingestion of alcoholic beverages, ethanol is converted into acetaldehyde, which is then oxidized to the non-toxic compound acetate. However, this mechanism does not explain why alcohol consumption preferentially exerts a local carcinogenic activity, because most of the ingested ethanol is not converted to acetaldehyde until it reaches the liver. The low amounts of acetaldehyde produced before ethanol reaches the liver might be sufficient to induce mutations in the cells lining the oral cavity, pharynx, larynx and esophagus. However, these possible mutations are eliminated when the cells lining these tissues are replaced by new cells during physiological tissue renewal. We cannot forget that carcinogenesis requires the multistep accumulation of DNA changes over years or decades, and that these cells do not live long enough to accumulate these DNA changes [5]. We recently hypothesized that the cytotoxic activity of ethanol could explain why alcohol consumption preferentially increase the risk of these cancers [5]. In short, the cytotoxicity of ethanol on the cells lining the oral cavity, pharynx, larynx and esophagus forces the stem cells located in deeper layers to divide more often than usual to replace the damaged epithelia. The accumulation of cell divisions in stem cells promoted by regular alcohol consumption leads to a variety of cancer related errors (e.g., mutations arising during DNA replication) that increase their risk of malignant transformation. The local carcinogenic effect of ethanol decreases or ends when alcoholic beverages reach a non-empty stomach, because the stomach content dilutes ethanol to non-cytotoxic concentrations [5]. However, direct evidence of the cytotoxicity of short-term exposures to ethanol on human epithelial cells was lacking. Here, we provide such evidence, analyze recent data that strongly support the mechanism of carcinogenesis we propose, and discuss a simple preventive strategy to reduce the high risk of cancer of the oral cavity, pharynx, larynx and esophagus in alcohol users.

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Materials and Methods

The human keratinocyte cell line HaCaT (Cell Line Service; L#300493-4212) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) at 37°C in a humidified atmosphere containing 5% CO_2. DMEM was supplemented with 50 µg/mL penicillin, 50 µg/mL streptomycin and 10% fetal bovine serum. Cell culture reagents were obtained from Biowest and from Thermo Fisher Scientific. Ethanol absolute (≥99.8%, AnalaR NORMAPUR, ACS, Reag. Ph. Eur.) was obtained from VWR Chemicals.

Exponentially growing cells were seeded into 96-well plates until they formed a monolayer. Then, solutions of PBS or culture medium (DMEM) containing specific concentrations of ethanol were added and immediately removed from the cells (exposure times were between 2 and 3 seconds). After a 20-h recovery period in ethanol-free medium, cell viability was estimated with the MTT assay. This colorimetric technique is based on the capacity of viable cells to transform the MTT salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a formazan dye. After treatments and recovery periods, the medium was removed, and 125 µL MTT (1 mg/mL in medium) was added to each well for 4 hours. Then, 80 µL 20% SDS in 0.02 M HCl were added to the plates and were incubated overnight at 37°C. Finally, optical densities were measured at 540 nm on a multi-well plate spectrophotometer reader [6]. All data were averaged from at least three independent experiments and were expressed as means ± standard error of the mean (SEM).

Results and Discussion

The lining of the oral cavity, pharynx, larynx and esophagus is mainly formed by squamous epithelium made of keratinocytes. The soft tissues of the oral cavity and esophagus are covered by a non-keratinized stratified squamous epithelium, whereas regions associated with mastication (i.e., the gingiva and hard palate) are covered by a keratinizing epithelium resembling that of the skin epidermis [7]. We therefore selected a human keratinocyte cell line for our experiments. After choosing several concentrations of ethanol typically present in popular alcoholic beverages (beer, wine and some distilled alcoholic beverages), we exposed the cells for only 2-3 seconds to PBS or culture medium containing these concentrations of ethanol (5%, 10%, 15% and 40%). A drastic reduction in cell viability was observed for the higher ethanol concentration (Figure 1A and B). To more closely simulate alcohol consumption, we exposed the cells to the ethanol solutions for 2-3 seconds every minute during five consecutive minutes. A marked cytotoxic effect was observed for ethanol 15%, but not for ethanol 10% (Figure 1C and D). Further experiments revealed that ethanol started to cause a concentration-dependent cytotoxic effect between 10% and 15% (Figure 1E and F). Similar results were obtained in another non-malignant cell line (immortalized human fibroblastic hTERT-BJ cell line) commonly used in our laboratory (results not shown). Our data clearly show that short-term exposures to concentrations of ethanol present in alcoholic beverages are cytotoxic to human epithelial keratinocytes (Figure 1).

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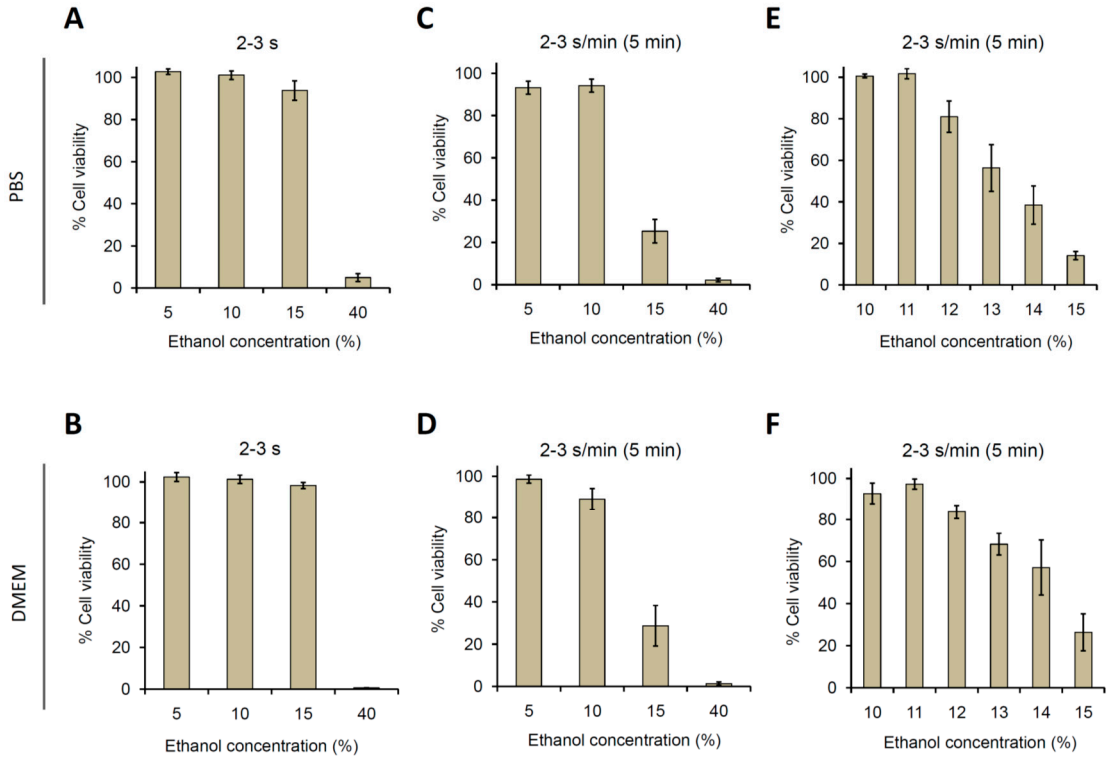

Figure 1. Short-term exposures to ethanol concentrations found in alcoholic beverages are cytotoxic to human epithelial keratinocytes. HaCaT cells were exposed to PBS or culture medium (DMEM) containing several concentrations of ethanol; these ethanol solutions were added and immediately removed from the cells (exposure times were between 2 and 3 seconds). In A and B, cells were exposed only once to the ethanol solutions. In C, D, E and F, cells were exposed once per minute for five minutes. After treatments, cells were grown in fresh medium and cell viability was estimated with the MTT assay.

If we regularly drink alcoholic beverages containing cytotoxic concentrations of ethanol, the stem cells located in deeper layers of the affected tissues will need to divide more often than usual to renew the damaged cells to maintain tissue function. It has been estimated that the stem cells of the oral cavity, pharynx, larynx and esophagus divide approximately every 2-3 weeks [7,8]. These division rates will probably increase if the ingestion of cytotoxic concentrations of ethanol shortens the life of the cells lining these tissues. The accumulation of cell divisions in stems will increase their risk of malignant transformation, because cell division is associated with a variety of cancer-promoting errors. For example, cell division generates spontaneous mutations arising during DNA replication [9]; it has been estimated that three mutations may occur every time a normal stem cell divides [10,11]. Cell division can also generate chromosome aberrations occurring during mitosis [12-16]; chromosome segregation errors can lead to mutations and chromosome rearrangements that integrate into the genome [13,14]. These DNA alterations can affect oncogenes and tumor-suppressor genes and disorder genetic programs controlling stem cell behavior and fate. In addition, cell division exposes the DNA of the cell to the genotoxic activity of DNA-damaging agents, such as

acetaldehyde and tobacco carcinogens. During cell division, the DNA unwinds to be copied during DNA replication and the nuclear membrane disintegrates during mitosis; these cellular events facilitate the interaction between DNA-damaging agents and the DNA of the cell. Therefore, the accumulation of cell divisions in stem cells triggered by alcohol consumption can promote the accumulation of DNA alterations in stem cells and increase their risk of malignant transformation [5].

Cumulative evidence strongly suggests that cancer is the end-result of the accumulation of cell divisions in stem cells [17]. First, most cancers originate in self-renewing tissues, that is, in tissues that accumulate many stem cell divisions throughout life. Cancer almost never arises from organs and tissues composed of cells that rarely divide, even though these cells are also exposed to naturally-occurring DNA damage and to environmental carcinogens. The variation of cancer incidence among tissues with different renewal capacities are striking; for example colon cancer is diagnosed about 100,000 times more frequently than heart cancer [18,19]. Furthermore, cancer incidence increases dramatically with age in tissues that accumulate cell divisions with age. For example, cancers of the oral cavity, pharynx, larynx and esophagus are diagnosed almost one hundred times more frequently in people over 60 years old than in people under 30 [5,18]. Finally, a recent study revealed a highly positive correlation between the number of stem cell divisions occurring in a tissue during a person's life and the risk of being diagnosed with cancer in that tissue (Spearman's rho = 0.81; $P < 3.5 \times 10^{-8}$) [8]. Although this study was confined to the U.S. population, the authors have recently confirmed that this striking correlation stands in 69 countries representing over half of the world's population [20]. Altogether, the evidence strongly suggests that the main biological cause of cancer is the accumulation of cell divisions in stem cells. The accumulation of cell divisions in stem cells not only drives the accumulation of the DNA alterations required for carcinogenesis, but also the formation and growth of the abnormal cell populations that characterize the disease [17].

The large differences in cancer incidence among tissues with different renewal capacities indicate that cell death occurring during physiological tissue renewal is crucial for carcinogenesis. Once our tissues are formed and we stop growing, cell death occurring during normal tissue renewal becomes the main reason for cell division and the main risk factor for cancer [17]. This implies that cell death triggered by pathological factors (e.g., tissue injury, infections, and inflammation) or by environmental factors (e.g., drinking alcoholic beverages containing cytotoxic concentrations of ethanol) can also promote carcinogenesis. Pathological and environmental cytotoxic factors will play a major role in carcinogenesis if they become chronic or persistent, because they will trigger a continuous renewal of the damaged tissue. These factors do not need to cause an acute cytotoxic effect to increase the risk of cancer. They simply need to cause sufficient damage to reduce the lifespan of the cells that need to be replaced by the stem cells. Stem cells will have to divide more often than usual, and they will accumulate an extra number of cancer-promoting errors that will increase their risk of malignant transformation [17].

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In our opinion, the accumulation of cell divisions in stem cells promoted by regular alcohol consumption explains the high risk of cancer of the oral cavity, pharynx, larynx and esophagus in alcohol users. This explanation has an important implication for preventing these cancers. It is widely accepted that the only way to reduce the risk of cancer in alcohol users is to limit the amount of alcohol. However, because alcohol consumption causes addiction, this recommendation is not always easy to follow. Choosing alcoholic beverages containing non-cytotoxic concentrations of ethanol, or diluting ethanol to non-cytotoxic concentrations, may be an effective and easy-to-follow recommendation to reduce the high risk of these cancers in alcohol users. This reduction may be particularly marked in drinkers who also smoke, because cell division triggered by cytotoxic concentrations of ethanol exposes the stem cell of these tissues to local and systemic tobacco carcinogens [17,21]. Unfortunately, most epidemiological studies assessing the influence of alcohol consumption on the risk of cancer have considered the amount of alcohol consumed but not the concentration at which ethanol is ingested. However, evidence supporting our proposal is already available [22]. In a case-control study conducted in Puerto Rico, the risk of oral and pharyngeal cancer among people who drank undiluted liquor was consistently higher than for those who drank similar amounts of diluted liquor [22]. In smokers, the risk of these cancers was also much higher for people who drank undiluted liquor than for those who drank similar amounts of diluted liquor [22]. Opting for alcoholic beverages containing non-cytotoxic concentrations of ethanol, or diluting ethanol to non-cytotoxic concentrations, may reduce or even abolish the known synergistic effect between alcohol and tobacco on the risk of cancers of the oral cavity, pharynx, larynx and esophagus [23-26].

The concentrations of ethanol required to induce cytotoxicity in our *in vitro* experiments may be somewhat different from those required to induce cytotoxic effects in humans. Epidemiological studies are needed to determine the threshold concentration of ethanol below which the local carcinogenic activity of ethanol can be avoided. These studies need to use data from people who regularly drink beverages with a known final concentration of ethanol. In the meantime, diluting one measure of whisky or vodka (40% ethanol) with at least three measures of a non-alcoholic mixer may be an effective way to reduce the high risk of cancer of the oral cavity, pharynx, larynx and esophagus in alcohol users. This recommendation may be particularly important for drinkers who also smoke.

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