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Genotoxicity assessment of vaccine adjuvant squalene

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ABSTRACT

The genotoxic potential of the vaccine adjuvant Squalene was assessed by the chromosomal aberrations (CAs), sister chromatid exchanges (SCEs) and micronucleus (MNs) tests in human lymphocytes and comet assay in both human and rat lymphocytes. Five different concentrations of squalene (1250–20,000 μg/ml for human lymphocytes and 0.07–1.12 mg/kg for rat lymphocytes) were studied. Squalene did not affect the CAs and MN frequency, in all treatments *in vitro*. A significant increase in SCEs was observed in almost all concentrations at 24 h treatment. Squalene did not affect significantly the comet tail length (CTL) (except 2500 μg/ml) and comet tail intensity (CTI) at all treatments *in vitro*. In rats, squalene significantly increased and decreased CTL and CTI in some doses. Although there are increasing and reduction in the effect, squalene cannot be regarded as genotoxic in human lymphocytes. However, further *in vivo* studies are required to be sure on the effect.

1. Introduction

Squalene is a polyunsaturated triterpene containing six isoprene units (Mesa and Fernández, 2004). It has been used for various applications, especially in vaccine as adjuvant (Mesa and Fernández, 2004; Reddy and Couvreur, 2009). An immunological adjuvant is a substance employed to increase or to modulate the immune response against an antigen. An ideal adjuvant is therefore expected to increase the potency of the immune response while remaining non-toxic and safe for the host (Mesa and Fernández, 2004). Because of its significant dietary benefits, biocompatibility, inertness, and other advantageous properties, squalene is also used as an excipient in pharmaceutical formulations for disease management and therapy (Reddy and Couvreur, 2009). Squalene is widespread in nature, especially in olives, shark liver oil, wheat germ, and rice bran. Thus, in addition to being synthesized within cells, it is consumed as an integral part of the human diet (Liu et al., 1976; Newmark, 1997; Reddy and Couvreur, 2009). In humans, squalene is synthesized in the liver and the skin, transported in the blood by very low density lipoproteins (VLDLs) and low density lipoproteins (LDLs), and secreted in large quantities by the sebaceous glands (Koivisto and Miettinen, 1988; Stewart, 1992; Reddy and Couvreur, 2009).

According to the literature searches, genotoxicity studies related to squalene are limited. The study of raw shark liver oil containing squalene to increase the frequency of micronucleus was determined. Authors reported that the crude liver oils of three species of sharks are genotoxic in human lymphocytes *in vitro* (Bartfai et al., 2000). On the other hand, several researchers explained that squalene has non-genotoxic properties. These studies indicated that squalene has curative effects on DNA damage using sister chromatid exchange and micronucleus test systems (Fan et al., 1996; O'Sullivan et al., 2002). In addition squalene acts as a protective agent and has been shown to decrease chemo- and radiotherapy-induced side-effects (Xu et al., 2005; Reddy and Couvreur, 2009; Narayan et al., 2010). Das et al. (2008) reported that squalene selectively protected mouse bone marrow progenitors against cisplatin and carboplatin-induced cytotocity *in vivo* without protecting tumor growth. These results are in agreement with Narayan et al.'s (2010) report. They assessed the protective effect of squalene against the genotoxicity of the chemotherapeutic agent doxorubicin using micronucleus and comet assay. They showed that squalene did not induce any significant DNA damage by itself. They also suggested that the complementary use of squalene with doxorubicin will be beneficial to reduce the adverse effect of doxorubicin in cancer chemotherapy.

To our knowledge there have been no published studies investigating chromosomal aberrations (CAs), sister-chromatid

Abbreviations: CAs, chromosomal aberrations; SCEs, sister chromatid exchanges; MN, micronucleus; CTL, comet tail length; CTI, comet tail intensity; RI, replicative index; MI, mitotic index; NDI, nuclear division index; MMC, mitomycin-C.

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exchanges (SCEs) and micronucleus (MNs) frequencies and DNA damage in human lymphocytes and/or rat lymphocytes treated with squalene, *in vitro* and *in vivo*, respectively. Determination of the squalene genotoxicity is fairly important for human health since it is extensively used in vaccines and drugs. This paper aims to determine whether squalene has genotoxic potential *in vivo* and *in vitro*. In the present study, we used various genotoxicity tests such as, comet assay (single cell gel electrophoresis-SCGE) both *in vivo* and *in vitro* and, chromosomal aberration, sister chromatid exchange and micronucleus tests *in vitro*. These test systems were selected as they are frequently used and provide sensitive assays to measure mutagenicity, clastogenicity, and potential carcinogenicity of chemicals (Yüzbaşıoğlu et al., 2008; Yılmaz et al., 2009; Balasubramanyam et al., 2009; Mamur et al., 2010).

2. Materials and methods

2.1. Chemicals

The test substance squalene (minimum 98% purity) was purchased from Sigma (CAS. No: 111-02-4) and dissolved in physiological saline. The chemical structure, molecular formula and molecular weight of squalene are shown in Fig. 1. The other chemicals cytochalasin-B (CAS. No: 14930-96-2), mitomycin C (CAS. No: 50-07-7), bromodeoxyuridine (CAS. No: 59-14-3), NaCl (CAS. No: 7647-14-5), colchicine (CAS. No: 64-86-8) were obtained from Sigma. DMSO (CAS. No: 67-68-5), NaOH (CAS. No: 1310-73-2), tris (CAS. No: 77-86-1), EDTA (CAS. No: 6381-92-6), triton X-100 (CAS. No: 9002-93-1), low melting agarose (CAS. No: 9012-36-6), normal melting agarose (CAS. No: 9012-36-6), EtBr (CAS. No: 1239-45-8) and H_2O_2 (CAS. No: 7722-84-1) were obtained from Applichem. PBS (CAS. No: L1825), Biocoll (CAS. No: L 6115) and Chromosome Medium B (CAS. No: F5023) were obtained from Biochrom AG.

Molecular formula: 2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene, molecular weight: 410,73.

2.2. Human lymphocyte culture for chromosome aberration, sister chromatid exchange and micronucleus tests

For *in vitro* genotoxicity tests, human peripheral lymphocytes were used as the test system. Therefore, peripheral blood was obtained from two healthy (1 male and 1 female) non-smoking donors (of ages 24–26 years), they had no medication within the prior 3 weeks and no radiological examination within the prior 3 months. Whole blood (0.2 ml) was added to 2.5 ml Chromosome Medium B supplemented with 10 μg/ml bromodeoxyuridine. The cultures were incubated at 37 °C for 72 h. The cells were treated with 1250, 2500, 5000, 10,000, 20,000 μg/ml concentrations of squalene for 24 h and 48 h. In addition, a negative (physiological saline) and a positive (mitomycin-C: MMC) controls were also used. The methods of Evans (1984) and Perry and Thompson (1984) were followed in CA and SCE tests with minor modifications (Yüzbaşıoğlu et al., 2006). For the SCE assay, the slides were stained with Giemsa, according to Speit and Houpter's (1985) method, with some modifications (Yüzbaşıoğlu et al., 2006).

To evaluate the chromosomal aberrations, 200 well spread metaphases (100 metaphases from each donor) were scored blindly, for each treatment. The mean frequency of abnormal cells and the number of CAs per cell (CAs/cell) were calculated. The mitotic index (MI) was determined by scoring of 1000 cells from each donor.

In order to score SCEs, 25 second-division metaphases (M2) were analyzed blindly for each donor. In addition to SCEs, cells were analyzed for the relative frequency of first division metaphases (M1), second-division metaphases (M2), and third-and subsequent division metaphases (M3). Replication index (RI) is the average number of replications completed metaphase cells and is calculated as follows: $[(1 \times M1) + (2 \times M2) + (3 \times M3)]/N$ (N = number of observed cells) (Schneider and Lewis, 1981).

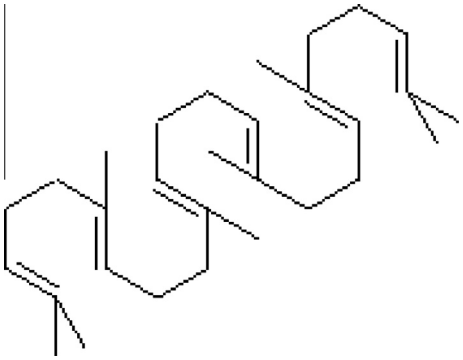

Fig. 1. The chemical structure of squalene.

Micronucleus preparation was performed according to the procedures of Fenech (2000) and Palus et al. (2003). Whole blood (0.2 ml) was added to 2.5 ml of Chromosome Medium B. The peripheral lymphocytes were incubated at 37 °C for 72 h and exposed to squalene at 1250, 2500, 5000, 10,000 and 20,000 μg/ml concentrations during the last 48 h. 44 h from the initiation, cytochalasin B was added to block cytokinesis.

Totally two thousands binucleated cells (1000 cells per donor) for each treatment of squalene were examined blindly, following the scoring criteria adopted by the Human Micronucleus Project (Bonassi et al., 2001). Five hundred cells per donor (totally 1000 cells) were scored to evaluate the nuclear division index (NDI). NDI was calculated using the following formula: $[(1 \times N1) + (2 \times N2) + (3 \times (N3 + N4))]/N$; where $N1$ to $N4$ represent the number of cells with one to four nuclei and N is the total number of viable cells scored (Surrales et al., 1995).

2.3. Comet assay

2.3.1. In vitro test

In this study the alkaline version of the comet assay was performed according to Singh et al. (1988) with a slight modification (Mamur et al., 2010). Peripheral blood was obtained from two healthy (1 male and 1 female) non-smoking donors (of ages 24–26 years), they had no medication within the prior 3 weeks and no radiological examination within the prior 3 months. Lymphocytes were isolated using Biocoll separating solution. To detect the viability of cells, trypan blue exclusion test was used. Cell viability was >98%. Isolated human lymphocytes were incubated with different concentrations of squalene (1250, 2500, 5000, 10,000 and 20,000 μg/ml) at 37 °C for 1 h. A negative (physiological saline) and a positive control (100 mM H_2O_2) were also maintained.

The slides were incubated for 20 min ice-cold electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH > 13), followed by electrophoresis at 25 V, 300 mA for 20 min. Each slide was stained with 50 μl of 20 μg/ml ethidium bromide. The slides were examined using a fluorescent microscope (Olympus) equipped with an excitation filter of 546 nm and a barrier filter of 590 nm at 400× magnification. The tail lenght (μm) and tail intensity (%) of 100 comets on each slide (a total of 200 comets per concentration) were examined, using specialized Image Analysis System (''Comet Assay IV'', Perceptive Instruments Ltd., UK).

2.3.2. In vivo test

In this study, comet assay was performed on Wistar rats (10–12 weeks old) that were procured from Refik Saydam National Public Health Agency, Experimental Animal Unit (Ankara, Turkey). Rats were kept in separate cages in an experimental room under controlled conditions of temperature (22 ± 2 °C) and humidity (50–60%) with feed and water being available ad libitum. Lighting was controlled to provide 12 h artificial light followed by 12 h darkness. Animal experiments have been complied with the principles of the local ethics committee.

Rat blood samples taken from two different groups were used in *in vivo* Comet assay. Group 1 was the treatment group studied 1 day after the squalene injection. Group 2 was studied 14 days after squalene injection. Five dose levels of squalene (0.07 mg/kg, 0.14 mg/kg, 0.28 mg/kg, 0.56 mg/kg, 1.12 mg/kg) were given to rats subcutaneously for each treatment groups. In addition, an untreated control and a positive control (mitomycin-C, 2 mg/kg) were also used to test the validity of the assay for all treatment groups. Five rats were used in each application groups (totally 35 rats). Concentrations were determined based on the amount of squalene in human vaccines.

For *in vivo* comet assay, approximately 100 μl whole blood was collected from rat's tail vein into lithium–heparin tubes. Due to the intensity of cells, blood samples were diluted and suspended with phosphate buffer (pH: 7.4) in 1:1 ratio and then centrifuged (Smith et al., 2008). Lymphocytes were isolated by Biocoll separating solution. After the isolation step, lymphocytes were resuspended in PBS (phosphate buffered saline). Afterwards, the protocol for *in vitro* comet assay described above was applied. Quantification of DNA breakage was realized using Comet Image Analysis System (''Comet Assay IV'', Perceptive Instruments Ltd., UK). At least 300 comets for each experimental group were recorded as tail length and tail intensity.

2.4. Statistical analysis

For data evaluation, the z-test was used for the percentage of abnormal cells, CA/cell, MI, RI, NDI, MN assays. The t-test was applied for SCEs and comet assay results to determine the statistical difference between treated and untreated samples. Dose–response relationships were determined from the correlation and regression coefficients for the percentage of abnormal cells, CA/cell, SCE, mean MN and DNA damage.