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RESEARCH ARTICLE

EVALUATION OF CYTOTOXIC AND GENOTOXIC EFFECTS OF SAXAGLIPTIN

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Abstract

Saxagliptin (SAX) is an oral drug that is a hypoglycemic (between an anti-diabetic agent) dipeptidyl peptidase-4 inhibitor. In this study, cytotoxic, genotoxic effects and DNA damage of SAX on human lymphocytes were investigated. For this purpose, Single Cell Gel Electrophoresis (SCGE), Micronucleus (MN) and Mitotic Index (MI) tests were used. Based on the daily doses of SAX, 0.017, 0.035, 0.07, 0.14 µg/mL concentrations used for the study. SAX significantly reduced the MI only at the highest concentration (0.14 µg/mL) for 24 hours, and 0.07- and 0.14-µg/mL concentrations for 48 hours. SAX did not cause a statistically significant change in MN frequency (except for concentration 0.14 µg/mL). In the SCGE test, a statistically significant increase of comet tail length was observed at 0.07- and 0.14-µg/mL concentrations. SAX did not cause a statistically significant change in comet tail moment and tail intensity (except for concentration 0.14 µg/mL). As a result, SAX caused statistically differences in the SCGE, MI and MN tests only at the highest concentrations that are not recommended commercial use (except for tail length, 0.035 µg/mL). When the results of all these studies are evaluated together, it can be said that SAX has no aneugenic, mutagenic and clastogenic effects at daily doses in *in vitro* studies on human lymphocytes.

Introduction:-

The American Diabetes Association (2004) stated Diabetes mellitus as a group of metabolic diseases defined by hyperglycemia and caused by disorders in insulin action, insulin secretion, or both insulin secretion and action. Diabetes can cause damage to the nerves, eyes, kidneys, blood vessels, and heart, in due course (WHO, 2021). Diabetes is divided into three classes as Diabetes mellitus Type 1, Diabetes mellitus Type 2, other specific types of diabetes and gestational diabetes (ADA, 2004; Galtier, 2010). As reported by the World Health Organization (2021), the number of people with diabetes reached 422 million in 2014 and caused an estimated 1.5 million deaths in 2019. In addition, the world prevalence of Diabetes mellitus among adults (20-79 years old) is estimated to rise to 439 million by 2030 (Shaw et al., 2010).

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Incretins, which are enteroendocrine hormones, stimulate insulin secretion and also regulate glucagon secretion in a glucose-dependent manner (Idorn et al., 2014). The incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are released into the bloodstream in response to food intake and are secreted from L and K cells, which are distributed throughout the gastrointestinal tract (Idorn et al., 2014; Chia and Egan, 2008). Both GLP-1 and GIP lose their effect by degradation within minutes by the enzyme dipeptidyl peptidase IV (DPP-4) (Amori et al., 2007; Drucker and Nauck,2006).

DPP-4 inhibitors, which used to treat diabetes, are a type of potent anti-glycemic agents (Zeng et al., 2019). SAX is one of the DPP4 inhibitors that improves glycemic control by preventing the inactivation of GLP-1 and GIP hormones, causing an increase in GLP-1 level and insulin secretion, and a decrease in postprandial glucagon and glucose levels (Dhillon and Weber, 2009; Yang, 2012).Considering its pharmacodynamic properties, SAX has been noted to be reversible, competitive, highly potent (Ki: 1.3 nM) and selective (EMA, 2008).

It is stated that most of the carcinogens cause mutations and DNA damage (Jackson and Bartek, 2009; Turkez et al., 2017). Therefore, genotoxicity should be well evaluated throughout the safety assessment process. Genotoxicity is a worldwide public concern due to the carcinogenic effects of chemicals and the heritability of these effects. Therefore, genotoxicity tests have become a requirement in countries (Turkez et al., 2017; Gonzalez and Kirsch-Volders, 2016; Annangi et al., 2016; Nersesyan et al., 2016). Despite the importance of genotoxicity studies, there is little information about the genotoxicity of SAX. Although, it has been widely used in many countries in recent years(Dhillon and Weber, 2009; Yang, 2012). To our knowledge, there is very little information about the genotoxicity and cytotoxicity of SAX in the literature. Therefore, the aim of this study was to test the potential genotoxicity and cytotoxicity of SAX in human lymphocytes by *in vitro*SCGE, micronucleus (MN) and mitotic index (MI) test.

Material and Methods:-

Chemical used in the study

The commercial form of SAX was purchased from the pharmacy. SAX has been isolated from commercially available drugs. For this, the drug tablets were ground into powder by pounding in sterile mortar. The powder form of the drug was dissolved in 100 mL of methanol for 20 hours in a magnetic stirrer. Subsequently, after filtering with filter paper, the solvent was removed via the evaporator and the substance was obtained pure.

In this study, based on the daily doses of SAX of 2.5 and 5 mg, the daily doses as well as the half of the lowest concentration (1.25 mg) and twice the highest concentration (10 mg) were evaluated. We accepted the weight of a normal individual as 71 kg in our study. An equivalent concentration of 0.14 µg/mL was calculated as the dose/kg ratio, corresponding to 10 mg/71 kg. Hence, 0.14, 0.07, 0.035 and 0.017 µg/mL were used in our study. Positive (0,2 µg/mL of Mitomycin-C for MI and MN, 3.4 µg/mL of H_2O_2 for SCGE) and negative (distilled water) controls were also included as control groups in the studies.

Tests performed in the study

This study was approved by CanakkaleOnsekiz Mart University Clinical Research Ethics Committee (No: 2011-KAEK-27/2020-E.2000110033).

In each of the tests (MI, MN, SCGE) to be performed, blood was taken from 4 volunteers, 2 men and 2 women. Blood samples were taken from healthy volunteers aged 20-25, who did not smoke, do not use alcohol, do not have a chronic disease, did not use any medication in the last week, had not been exposed to any radiation in the last 6 months.

Mitotic Index (MI) Test

Whole blood (0.2 mL) from 4 healthy volunteers who were not exposed to any genotoxic agents was incubated for 72 hours at 37°C in culture tubes containing 2.5 mL of chromosome medium B. The determined concentrations of SAX were added to the culture tubes 24 hours and 48 hours after the start of the culture, and colchicine (0.06 g/mL) was added to each culture at the 70^{th} hour of culture. At the end of the incubation, the cultures were centrifuged at 1200 rpm for 10 min and the cells were harvested. The harvested cells were incubated in a hypotonic solution (KCl, 0.075 M) at 37 °C for 30 min and then in cold methanol-acetic acid (3:1) for 45 min in 4 °C for fixation. This fixation step was performed in 3 repetitions. Cells, collected after final fixation, were prepared by dripping onto slides. The dried preparations were stained with 5% Giemsa dye (pH: 6.8) for 20-25 min, and after being passed

through distilled water 3 times, they were turned into a permanent preparation and examined under a light microscope. In determining MI, a total of 12000 cells for each concentration, 3000 cells for each individual for all treatments, were examined. The MI was determined by calculating the ratio of the number of dividing cells to the total cells as a percentage.

Micronucleus (MN) Test

Blood taken from healthy individuals was transferred to culture tubes containing 2.5 mL of medium, as 0.2 mL. Tubes were cultured at 37 °C for 72 hours. At 24th hours of culture, predetermined concentrations of SAX were added to the cultures. Cytochalasin-B (5.2 µg/mL) was added to the cultures at 44th hours of culture. At the end of the incubation, the cultures were centrifuged at 1000 rpm for 10 min and the cells were harvested. The harvested cells were incubated in a cold hypotonic solution (KCl, 0.075 M) at 4 °C for 5 min and then in cold methanol-acetic acid (3:1) at 4 °C for 15 min for fixation. This fixation step was performed in 3 repetitions. 1% formaldehyde was added to the last 3:1 fixative solution. Cells, collected after final fixation, were prepared by dripping onto slides. The dried preparations were stained with 5% Giemsa dye (pH: 6.8) for 10-13 min, and after being passed through distilled water 3 times, they were turned into a permanent preparation and examined under a light microscope. MN frequencies were determined by examining 1000 binucleate cells in each individual and a total of 4000 binucleate cells for each concentration in the permanent preparations. In addition, for each concentration, the nuclear division index was determined by counting 500 cells from each individual and 2000 cells in total. The nuclear division index (NDI) was calculated as follows: [1 × N1] + [2 × N2] + [3 × (N3 + N4)]/N. N1–N4 refers to the number of cells containing 1-4 nuclei, respectively, while N refers to the total number of cells analyzed.

Comet (SCGE) Test

The SCGE test was performed according to the original procedure (Singh et al., 1988) with some modifications. To perform the SCGE Test, 900 µL of PBS was placed in ependorfs and 100 µl of blood from healthy volunteers who were not exposed to any genotoxic agent was added to each ependorf. After the blood and PBS were suspended and kept on ice for 10 min, lymphocytes were collected by adding lymphopreb to the ependorfs. The obtained lymphocytes were cultured with predetermined concentrations of SAX and negative and positive controls at 37 °C for 1 hour. After culture, eppendorfs were centrifuged at 3000 rpm for 5 min. After centrifugation, the supernatant was discarded from the eppendorfs and resuspended by adding 100 µl of PBS to the lymphocytes. 75 µl of Low Melting Agar (0.65%) was taken, mixed rapidly with 100 µl of SAX-treated lymphocytes, and spread on slides previously coated with normal melting point agar (0.65%). Then, the slides were covered with a 24X60 mm coverslip and kept in a closed box at 4°C for 20-25 minutes. At the end of the time, the coverslips on the slide were removed and placed in the chalet containing the lysing solution and left at 4°C for 1 hour. After the lysis process, the slides were placed in the buffer in the electrophoresis tank and kept there for 20 minutes. At the end of the period, the preparations were subjected to electrophoresis. After electrophoresis, the slides were taken into a chalet containing neutralization buffer and kept there at 4 °C for 5 minutes, and the same process was repeated 2 times. At the end of the procedures, 50 µl EtBr was added to each slide and a coverslip was covered, and the stained preparations were examined under a fluorescent microscope at 40X magnification. For each concentration, a total of 400 cells, 100 cells from each donor (duplicate slides per treatment), were examined with a specialized image-analysis program (BS 200 ProP; BAB Imaging System, Ankara, Turkey) and the results were evaluated in terms of % tail density, tail length and tail moment.

Statistical Analyzes

While the z distribution test was used to determine whether the MI and MN in the test groups differed from the control groups, the students-t test was used in the evaluation of the SCGE. SPSS v. 22 package program (SPSS Inc., Chicago, IL, USA) was used to reveal the concentration-effect relationships of MI, MN frequency, comet tail length, tail density and tail moment.

Results:-

The MI(%) frequencies in human peripheral lymphocytes exposed to SAX of 24 and 48 hours are given in Table 1.

Table 1:- MI frequencies in human peripheral lymphocytes exposed to SAX.

Test substances	Treatment		Counted Cells	MI Frequency %
	Period (hours)	Concentrations (µg/mL)		

Negative Control	24 hours	0	12000	5.92±0.22
Positive Control (MMC)	24 hours	0.2	12000	2.95±0.15
Saxagliptin	24 hours	0.017	12000	5.39±0.21
		0.035	12000	5.85±0.22
		0.07	12000	5.61±0.21
		0.14	12000	5.22±0.20*
Negative Control	48 hours	0	12000	5.85±0.21
Positive Control (MMC)	48 hours	0.2	12000	1.81±0.12
Saxagliptin	48 hours	0.017	12000	5.7±0.21
		0.035	12000	5.63±0.21
		0.07	12000	5.27±0.20*
		0.14	12000	5.1±0.20*

*Statistically significant difference compared to control ($p<0.05$) (Z test)

According to these results, the MI decreased in all treatment concentrations with 24-hour exposure compared to the control. Of these reductions, only the highest concentration (0.14 µg/mL) was found to be statistically significant compared to the negative control ($p<0.05$). According to the results of 48-hour treatment of SAX in human lymphocytes, the MI decreased at all concentrations compared to the control, but this decrease was statistically significant only at two highest concentrations, 0.07 and 0.14 µg/mL ($p< 0.05$).

The MN and NDI frequencies in human peripheral lymphocytes exposed to SAX are given in Table 2.

Table 2:- MN and NDI frequencies in human peripheral lymphocytes exposed to SAX.

Test substances	**Treatment**		**Binucleated cells**	**MN frequencies**			**MN/Cell (%) ±SE**	**NDI±SE**
	Period (hours)	**Concent. (µg/ml)**		(1)	(2)	(3+)		
Negative Control	48	0.00	4000	6	1	0	0.2±0.07	1.44±0.27
Positive Control (MMC)	48	0.20	4000	564	82	16	19.4±0.62	1.20±0.24
Saxagliptin	48	0.017	4000	7	0	0	0.18±0.002	1.36±0.26
		0.035	4000	6	2	0	0.25±0.002	1.32±0.25
		0.07	4000	6	1	2	0.43±0.09	1.35±0.26
		0.14	4000	13	2	1	0.45±0.10*	1.25±0.25

*Statistically significant difference compared to control ($p<0.05$) (Z test)

When the data obtained were evaluated, MN frequency increased at all doses except the smallest concentration (Table 2). However, these increases were not statistically significant compared to the control, except for the highest concentration (0.14 µg/ml) ($p<0.05$).

It was determined that the NDI decreased with SAX treatment when compared to the control, but this decrease was not statistically significant ($p>0.05$).

In SCGE test, three different data were evaluated: tail length, tail intensity and tail moment. The DNA Damage on human peripheral lymphocytes exposed to SAX are given in Table 3.

Table 3:- Effects of SAX on DNA Damage.

Test substances	**Concent. (µg/mL)**	**Tail Length (µm)**	**Tail Moment**	**Tail Intensity (%)**
Negative Control	0.00	8.21 ± 0.62	5.78 ± 0.42	230.54 ± 0.37
Positive Control (MMC)	0.20	75.75 ± 2.27	72.91 ± 2.30	253.61 ± 0.31
Saxagliptin	0.017	8.77 ± 0.63	6.78 ± 0.40	231.62 ± 0.53
	0.035	9.80 ± 0.53*	5.93 ± 0.29	230.38 ± 0.43
	0.07	9.21 ± 0.84	6.47 ± 0.42	231.63 ± 0.51

	0.14	10.06 ± 0.59*	7.27 ± 0.51*	237.07 ± 0.40*

*Statistically significant difference compared to control ($p<0.05$) (Student t test)

In the present study, SAX significantly increased the comet tail length in human lymphocytes at concentrations of 0.035 and 0.14 µg/mL compared to the control ($p<0.05$). Likewise, it was determined that the comet tail intensity and tail moment increased only in the treatment of the highest concentration, 0.14 µg/mL, and this increase was statistically significant ($p<0.05$).

Discussion:-

Substances with positive genotoxicity tests are likely to be mutagens and carcinogens for humans, so these tests are frequently used in the estimation of carcinogenicity (EMA, 1998). Mutagens play role in many diseases, including mitochondrial and nuclear DNA damage, cardiovascular and neurodegenerative diseases, stem cell dysfunction, infertility, premature aging, chronic inflammatory conditions and diabetes mellitus type 2. Therefore, identification of potential mutagens is very important in the overall hazard assessment of drugs used in consumer and industrial applications, along with other chemicals (Jackson and Bartek, 2009; Turkez et al., 2017).

The advancement of DPP-4 inhibitors has recently been very hopeful for the treatment of type 2 diabetes (Barnett, 2006). SAX, a more potent and specific DPP-4 inhibitor than others, is given once daily (Augeri et al., 2005; Tahrani et al., 2009). In the present study, the genotoxic and cytotoxic effects of 0.14, 0.07, 0.035 and 0.017 µg/mL concentrations calculated on the basis of the daily doses of SAX, which is used extensively in the world, were investigated by SCGE, MN and MI tests.

Based on the current results, MI showed a statistically significant reduction only at the highest dose of the 24-hour SAX treatment and the two highest doses of the 48-hour treatment. According to these test results, it has been determined that SAX does not show cytotoxic effects in a short time at daily doses, but it may show cytotoxic effects in long-term use in excess of daily doses and at a concentration equivalent to the daily dose of 5 mg. Decreased MI, an indicator of cell cycle kinetics, reflects cytotoxicity (Unal et al., 2011). Accordingly, while no cytotoxic effect is observed in the 24-hour daily dose of SAX, it can be mentioned that the cytotoxic effect can be mentioned in the 48-hour daily use.

When we analyzed the MN and NDI results, it was determined that only the highest concentration of SAX, 0.14 µg/mL, showed a statistically significant difference. This concentration is higher than the equivalent concentrations of SAX daily doses. Therefore, it can be said that daily doses of SAX are not cytotoxic and aneugenic. The MN test is a simple test that is frequently used in different cell types and allows the detection of aneugens and clastogens. It is an internationally accepted, predictive test for cancer and has been used in genotoxicity studies, for a long while (Kirsch-Volders et al., 2011).

In the present study, DNA damage caused by SAX on human lymphocytes was investigated by the SCGE test. According to the results, 0.035 and 0.14 µg/mL concentrations of SAX showed a significant difference in comet tail length compared to the control. Likewise, when the results of the study were examined, a significant difference was observed in comet tail moment and comet tail density at only 0.14 µg/mL concentration of SAX. Based on the daily therapeutic doses of SAX; it was determined that there was a significant difference in tail length only at 0.035 µg/mL from the equivalent concentrations in our study. On the other hand, the equivalent concentrations of daily use doses of SAX do not statistically affect the DNA damage frequency in human lymphocytes *in vitro* in comet tail moment and comet tail density. The SCGE test, is a sensitive, rapid, inexpensive test that measures DNA damage at the cellular level in an alkaline environment (Qian et al., 2021; Koppen et al., 2018; Faust et al., 2004). SCGE test is one of the most effective, popular and widely used test applicable to many cell types to examine the mutagenic and genotoxic properties of carcinogens (Araldi et al., 2015; Yilmaz et al., 2014; Avuloglu-Yilmaz et al., 2017; Yuzbasioglu et al., 2018). To the best of our knowledge, the genotoxicity of SAX on human lymphocytes has not been investigated by the SCGE test until now.

Examining the EMA (2008) document, there was an information about SAX did not show mutagenic (Ames test), clastogenic (human lymphocytes) and genotoxic effects (*in vivo*/in rats). Similarly in NCBI (2004), there was an information about that SAX was not mutagenic or clastogenic (with/without metabolic activation in Ames test, in human lymphocytes/*in vitro*; MN test, DNA Repair Test in rats/*in vivo*; cytogenetics study in rat peripheral blood lymphocytes/*in vivo-in vitro*). However, there was no data on treatment duration and concentration ranges in these

documents. In parallel with EMA (2008) and NCBI (2004), in this study, it was determined that SAX did not have genotoxic, clastogenic, cytotoxic and aneugenic effects at daily doses.

In a study, the cytotoxicity, genotoxicity and oxidative damage potential of Linagliptin, one of the selective DPP-4 inhibitors, was investigated in human mononuclear blood cells. It was stated that there is no problem in the use of linagliptin in diabetic patients and that it can even provide protection against diabetic vascular and oxidative damage, in the aforementioned study (Cadirci et al., 2019). In another study, the cytotoxicity and genotoxicity of sitagliptin, one of the selective DPP-4 inhibitors, were examined and according to the results, it was stated that sitagliptin did not show genotoxic effect in low concentrations and short-term (24 hours) treatment, but it had genotoxic and cytotoxic effects at high concentrations and long-term treatment periods (Yuzbasioglu et al., 2018).

Conclusions:-

In conclusion, in this study, we examined the genotoxic and cytotoxic effects of SAX, one of the DPP4 inhibitors frequently used in the treatment of Type 2 diabetes, with SCGE, MN and MI tests. When the results of all these studies are evaluated together, it can be said that SAX has aneugenic, mutagenic and clastogenic effects only at high concentrations (over the amount of daily use) in *in vitro* studies on human lymphocytes. It has no cytotoxic or genotoxic effect at daily doses, except for comet tail length.

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