Journal of Health Sciences

RESEARCH ARTICLE Open Access

Cytogenetic effects of combination of tridecactide and met-enkephalin on lymphocytes of patients with multiple sclerosis

Maida Rakanovic-Todic[1]*, Slavka Ibrulj[2], Lejla Burnazovic-Ristic[1], Amra Catovic[2], Izeta Aganovic-Musinovic[2], Jasna Kusturica[1]

[1]Institute of Pharmacology, Toxicology and Clinical Pharmacology, Medical Faculty, University of Sarajevo, [2]Center for Cytogenetics and Molecular Medicine, Medical Faculty University of Sarajevo

ABSTRACT

Introduction: The met-enkephalin (1-5 Adrenorphin) and tridecactide (alpha-corticotropin 1-13) combination is used in the multiple sclerosis (MS) immuno-modulatory treatment. A testing of cytogenetic effects of met-enkephalin resulted in reductions of lymphocytic aberrations in the in the lymphocytes of the peripheral blood (PBL) of patients with immune-mediated diseases. The aim of this research is to evaluate the in vitro effects of the combination of the met-enkephalin and tridecactide on the number and the type of chromosome aberrations in PBL of the MS patients.

Methods: We used blood samples from seven female patients with the diagnosis multiple sclerosis based on a McDonald Diagnostic Criteria. The tested combination, met-enkephalin and alpha-ACTH 1-13 was added at three different concentrations and constant volume.

Results: Results showed that the combination of tested substances did not reduce the number of structural aberrations, although the treatment did not result in severe aberrations such as ring, fragmented, and dicentric chromosomes. Furthermore, it elicited an increase in the number of numerical aberrations and aneuploidy after the treatment with the test combo.

Conclusion: As the changes in ploidy significantly change the DNA as well as the biochemical cell phenotype, we concluded that more research in this field should be conducted, including both toxicological as well as the pharmacodynamic considerations.

Keywords: alpha-corticotropin 1-13; tridecactide; met-enkephalin; chromosome aberration; multiple sclerosis

INTRODUCTION

The met-enkephalin (1-5 adrenorphin) and tridecactide (alpha-corticotropin 1-13) combination is applied as the immunomodulatory treatment for Multiple Sclerosis (MS). Its application in clinical studies (1,2) significantly reduced the number of relapses (by 63%) and Expanded Disability Status Scale score in a treatment group (p=0.0001). The holder of the effects is tridecactide which is a

*Corresponding Author:Maida Rakanovic-Todic, Pharmacology, Clinical Pharmacology and Toxicology, Medical Faculty, University of Sarajevo, Čekaluša 90, 71 000 Sarajevo. E-mail: maida@dic.unsa.ba/maida.todic@mf.unsa.ba

Submitted: 20 October 2014 / Accepted: 6 April 2014

DOI: http://dx.doi.org/10.17532/jhsci.2014.237

UNIVERSITY OF SARAJEVO
FACULTY OF HEALTH STUDIES

deacetylated and deaminated alpha-melanocyte stimulating hormone (alpha-MSH). Endogenous alpha-MSH is acetylated in its N-terminal tail, and deaminated at the C-terminal. Alpha-MSH has antipyretic, anti-inflammatory and antimicrobial effects (3-5).

The peptide sequence of met-enkephalin and alpha-MSH exist within the same polypeptide precursor Proopiomelanocortin; however, met-enkephalin is not derived from this precursor - rather it is derived from proenkephalin (6). Enkephalin controls the secretion of alpha-MSH (7). Met-enkephalin in combination with deacetylated and deaminated alpha-MSH when individually applied in rats with ethanol-induced gastric damage elicits protective effects. When used in combination, a significant additive effect was achieved as well (8). The greatest effectiveness of this combination is achieved with the met-enkephalin and alpha-MSH-*like* of 51. Initial dosage for humans is 5-10 mg of met-enkephalin and 1-2 mg of alpha-MSH-*like*.

Patients with immune-mediated disease, including the MS patients, had aberrant lymphocyte clones that were stimulated by auto-antigens (9-11). A hypothesis regarding patients with auto-immune disease proposed that the treatment with certain active substances may normalize cytogenetic lymphocyte abnormalities what could suggest substances potential for therapeutic treatment. Met-enkephalin in the concentration of 1.2 μg/ml significantly reduced the number and the seriousness of chromosome aberrations *in vitro* (9). Furthermore, our testing of cytogenetic effects of tridecactide showed no significant reduction in the number of chromosome aberrations, while results implicated some tridecactide effect on the frequency of numeric aberrations (12).

The purpose of this research is to evaluate the effects of *in vitro* combination of met-enkephalin and tridecactide on the number and the type of chromosome lymphocytes aberrations in the peripheral blood in the patients with Multiple Sclerosis.

METHODS

Patients

We used the blood samples of seven female patients, with a diagnosis of MS in accordance with the McDonald Diagnostic Criteria for MS (2005 Revision), which were hospitalized due to relapse of the disease. Patients were not under the Interferon immunomodulatory treatment nor had received corticosteroids/pulse therapy over the previous six months.

Substances and concentrations

A combination of two active components, met-enkephalin and tridecactide (Enkorten®, Farmacija, d.o.o., Tuzla, Bosnia & Herzegovina), was tested. Met-enkephalin and alpha-ACTH 1-13, were added in concentration of 1.2 μg/ml and 0.24 μg/ml, respectively, into culture 2 (C2); or 120 μg/ml and 24 μg/ml, respectively, into culture 3 (C3). The combination was applied in the constant volume of 0.5 ml to achieve designated concentration per milliliter in the total culture volume (10 ml). The control culture was not incubated with the tested combination (C1). Test combination was dissolved in distilled water and added to culture immediately before placing the cultures into incubators.

Chromosomal analysis

A full blood sample was cultivated as per Moorhead et al. (1960) established techniques. The cultures were treated with phytohaemagglutinin and incubated at 37±0.5°C during 72 hours. Colcemid stock solution 25 mcg/ml (0.2 ml) was added two hours before completion of the incubation period. The chromosome aberrations were microscopically analyzed, after Giemsa staining. A number of structural and numerical aberrations were observed by analyzing 200 mitoses. Subsequent to the standard analysis of chromosome preparations, the identification of chromosomes included in the re-arranging after the coloring and application of the G-band technique was conducted.

Statistical analysis

The collected data was statistically processed in a computer program titled SPSS v.11 (*Statistical Package for Social Sciences©,* March 2004). We applied a non-parameter test for related samples, two-tailed *Wilcoxon Signed Ranks Test,* in order to test the difference between the findings on the

preparations in the control culture and the cultures incubated with different concentrations of the tested substance.

RESULTS

The age of female patients and the duration of the illness are listed in years, with an overview of the core diagnostic parameters (Table 1).

The core descriptive statistics for the total number of chromosomes is featured in Table 2 and it reflects structural aberrations on the cultured preparations. The observed structural aberrations are depicted on Figure 1.

The value of observed structural aberrations (gap, breaks, minute acentric fragments, ring, dicentric and fragmented chromosomes) within control group, culture 2 (conc. met-enkephalin 1.2 μg/ml and alpha-ACTH 1-13 0.24 μg/ml) and culture 3 (conc. met-enkephalin 120 μg/ml and alpha-ACTH 24 μg/ml).

The numerical analysis of the total number of chromosomes did not include chromosomes with gaps. This number was separately analyzed. Our consideration included one chromosome in the following structural aberration chromosome/chromatid break, acentric fragment, minute, ring chromosome, marker chromosome. Furthermore, our considerations included two chromosomes in the following structural aberrations dicentric chromosome and translocations. When the origin of the acentric fragment was available, it was classified as a break with

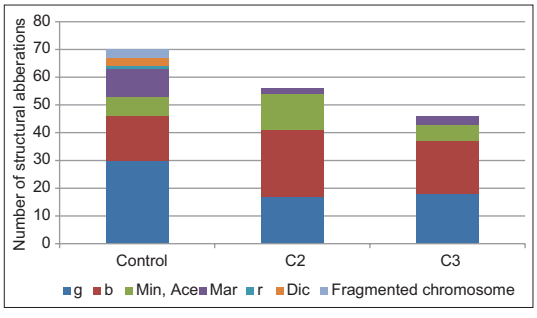

FIGURE 1. Frequency of the structural aberrations. Cell culture C2 was incubated with met-enkephalin and alpha-ACTH 1-13, in concentrations of 1.2 μg/ml and 0.24 μg/ml, respectively, while C3 was incubated with met-enkephalin and alpha-ACTH 1-13, in concentrations of 120 μg/ml and 24 μg/ml, respectively. Ace, acentric fragments; Min, minute acentric fragments; r, ring chromosome; Dic, dicentric chromosome; b, breaks; g, gap; Mar, marker chromosome

TABLE 1. Patient characteristics and the core diagnostic findings

Patient	Age (years)	Illness duration (years)	Number of hospitalizations	MRI*	CSF*	PEV*
P1	35	0.25	1	+	+	+
P2	60	17	3	+	-	-
P3	52	18	5	+	-	-
P4	48	6	2	-	-	-
P5	34	2	1	+	-	-
P6	37	0.5	1	+	+	+
P7	37	8	3	-	-	+

*MRI: Magnetic resonance imaging, CSF: Cerebrospinal fluid testing, PEV: Positive visual evoked potentials, +: Positive finding, -: Not conducted/data not existing

TABLE 2. The core descriptive statistic for detected aberrations and mitotic index

Culture code	N	$\bar{X}$±SD	Median	X_{min}	X_{max}
Total number of detected structural aberrations					
C1	7	6.14±2.67	5	4	12
C2	7	4.86±3.58	5	0	10
C3	7	3.71±2.06	4	1	6
Total number of detected numerical aberrations					
C1	7	3.29±2.69	2	1	9
C2	7	5.00±3.83	4	0	12
C3	7	6.29±3.15	6	3	12
Polyploidy					
C1	7	2.00±2.00	1	0	6
C2	7	2.57±1.62	2	0	5
C3	7	3.57±2.23	4	0	7
Endoreduplication					
C1	7	1.57±1.99	1	0	6
C2	7	1.43±1.13	2	0	3
C3	7	2.00±1.83	1	0	5
Aneuploidy					
C1	7	1.29±0.95	1	0	3
C2	7	2.43±3.51	1	0	10
C3	7	2.71±1.50	3	1	5
Mitotic Index					
C1	7	2.67±2.19	2.00	0.33	7.00
C2	7	3.09±1.09	3.00	1.33	4.67
C3	7	3.86±2.78	2.67	1.67	9.67

an associated acentric fragment. When the origin is unknown, it was classified as an acentric fragment.

Gaps, breaks and chromosome markers had the greatest representation. Only in C1, the ring, dicentric and fragmented chromosomes were detected.

There were no statistically significant difference recorded in the number of chromosomes included in structural aberrations (C2 p=0.293; C3 p=0.078). Additionally, there were no statistically significant difference in the number of chromosome/chromatid breaks (C2 p=0.167; C3; p=0.588), of gaps (C2 p=0.168; C3 p=0.225) and translocated marker chromosomes (C2 p=0.059; C3 p=0.059).

The frequency in chromosome aberrations was expressed in percentages (Table 3). Aberrations reviewed are gap, break, translocation, acentric fragments, dicentric and ring chromosome when the origin is available.

Chromosome the most frequently engaged in translocations was chromosome 14. In C1 two translocations were present between the chromosomes 2 and 14. In C2 preparations identified translocation between on chromosomes X and 14. In C3 preparations translocations were identified between chromosomes 4 and X, as well as 2 and 5.

The core descriptive statistic for the detected numerical aberrations is depicted in Table 2, while numerical aberrations are presented in Figure 2.

Value of numerical aberrations per culture within control group, culture 2 (conc. met-enkephalin 1.2 μg/ml and alpha-ACTH 1-13 0.24 μg/ml) and culture 3 (conc. met-enkephalin 120 μg/ml and alpha-ACTH 24 μg/ml).

For preparations form C3 was detected a statistically significant increase in the number of numerical aberrations (p=0.034) and in the number of aneuploidy (p=0.042), while there was no statistically significant difference for preparations from C2, respectively (p=0.104; p=0.197). Among the detected aneuploidy, trisomy and tetrasomy of chromosome X are the most dominating ones. No statistically significant difference was detected on the number of polyploidies (C2 p=0.458; C3 p=0.083) and of endoreduplications (C2 p=0.914; C3 p=0.480).

TABLE 3. Frequency of individual chromosome aberrations

Chromosome	C1 (%)	C2 (%)	C3 (%)
1	17.24	9.52	7.14
2	6.90	11.90	9.52
3	6.90	9.52	2.38
4	1.72	4.76	2.38
5	5.17	11.90	11.90
6	3.45	2.38	7.14
7	1.72	2.38	9.52
8	8.62	2.38	2.38
9	13.79	9.52	16.67
10	1.72	4.76	7.14
11	5.17	2.38	-
12	3.45	9.52	2.38
13	3.45	-	2.38
14	5.17	2.38	2.38
15	-	4.76	-
16	-	-	-
17	-	2.38	-
18	3.45	-	2.38
19	-	-	-
20	-	-	-
21	-	-	-
22	-	-	-
X	3.45	7.14	4.76
Unidentified chromosomes	n=5	n=1	n=3

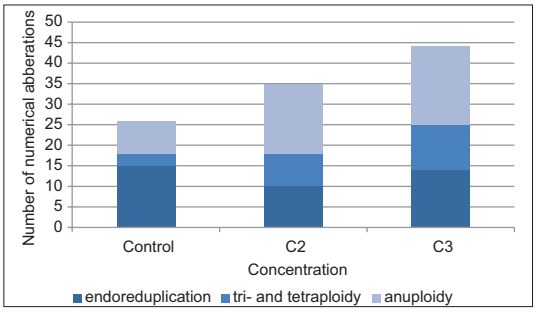

FIGURE 2. Frequency of numerical Aberrations. Cell culture C2 was incubated with met-enkephalin and alpha-ACTH 1-13, in concentrations of 1.2 μg/ml and 0.24 μg/ml, respectively, while C3 was incubated with met-enkephalin and alpha-ACTH 1-13, in concentrations of 120 μg/ml and 24 μg/ml, respectively.

The mitotic index was determined as the percent of lymphocytes in mitosis (M1 + M2) scored to 300 lymphocytes (Table 2).

No statistically significant difference between the mitotic index in C1 and incubated cultures with various concentrations of the tested substance (C2 p=0.735; C3 p=0.128), was detected.

DISCUSSION

Similarly to the Štambuk and associates (1998) research, we also detected disappearance of serious aberrations such as ring and dicentric chromosomes aberrations after the cultures had been incubated with test combination. Unlike to our results where the application of met-enkephalin of 1.2 μg/ml was tagged by a statistically significant reduction of the total number of structural aberrations, our research showed only a statistical significance tendency in a number of translocated marker chromosomes (C2 p=0.059; C3 p=0.059). The main methodological distinction in these two research is the duration of the culture incubation (48h i 72h). Also, Štambuk et al. (1998) observed the cell kinetics of five-day cell cultures that were added ^{3}H-thymidine. Cultures treated with met-enkephalin had a significant reduction in the number of cells reaching the third mitosis when compared to control cultures. It also detected a significant increase in the number of first metaphases (M1), and of the total number of cells in the second mitotic division that was the same as of the control culture.

In our research of cytogenetic effects of tridecactide (12) we detected no significant reduction in the number of chromosome aberrations, acentric fragments, ring chromosomes and dicentrics disappeared following treatment. The statistically significant increase in polyploidy and endoreduplications (p=0.047; p=0.033) was detected following incubation with the lower concentration of tridecactide resulted, and aneuploidy (p=0.026) following incubation with the higher concentration.

It was previously suggested that the distribution of spontaneous or induced breaks is not accidental. Two of the most common breaks (common fragile sites); FRA3B and FRA16D are tied to tumors of suppressor genes. Re and associates (2006) suggested that the fragile sites, via modulated gene expression, participate in the cell response to the DNA damage caused by oncogenes. It also suggested that the process of effective differentiation of T helper cells into the phenotype Th1 or Th2 occurs through several cell divisions, and that it includes cascade induction of transcription factor, while the importance of the genetic activities by T cells is attributed to epigenetic mechanisms (13-17).

In proportion to the absolute chromosome length, throughout our research, with the greatest percent of inclusion in chromosome aberration was of chromosomes 1 and 2. A higher percentage of chromosome 9 inclusion in aberrations is generally reflected in all tested cultures. For chromosome 5 treated by the tested combo, chromosome 6 in C3, for chromosome 8 in C1 and chromosome 12 in C2 this was also the case. Throughout our research, chromosome 14 was most frequently included in translocations.

Genetic tests support the hypothesis on polygenic predisposition toward multiple sclerosis. Region of the chromosome 6p21 is clearly tied to predispositions toward multiple sclerosis, as well as other regions such as 17q and 5q (18). In a wide genetic test, as the most interesting, the following regions were identified as well 1q, 6p, 9q and 16p as well as 3q and 5q (19).

We noticed a statistically significant increase in the number of numeric aberrations (p=0.034) and aneuploidy (p=0.042) on the C3 preparations. Polyploidy and aneuploidy are frequently present in the malignant cells. However, met-enkephalin used in both the animal and clinical studies suppressed the growth of malignant cells (20,21). Also, when higher dose of alpha-MSH was applied, no cell nevus was transformed into malignant cells (4). It is necessary to note that in experimental uses, other hormones, such as progesterone and follicle stimulating hormones showed aneugenic effects (22,23) as well as opioids, morphine and noscapine (24,25). Genotoxic effects of noscapine detected *in vitro* were not confirmed *in vivo.*

CONCLUSION

It can be concluded that the tested combination did not manifest significant clastogenic effects, nor that it showed particular protective potentials. Furthermore the treatment with the test combination did not record serious chromosome aberrations such as the ring chromosomes, acentric fragments,

and dicentric chromosomes. An increase in numeric aberrations and aneuploidy was recorded after the treatment with the test substances. The change in the cell ploidy significantly alters the DNA as well as the biochemical cell phenotype. We conclude that adequate interpretation of the detected findings reqiures more research in both the toxicological as well as the pharmacodynamic considerations.

ACKNOWLEDGEMENTS

The authors would like to thank to Farmacija society of limited responsibilities, Tuzla, for providing us with an opportunity to work in this area by donating the tested substances.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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