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ORIGINAL ARTICLE

Micronucleus evaluation for determining the chromosomal breakages after radionuclide synovectomy in patients with hemophilia

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Abstract
Objective To investigate the genotoxic effects of ^{90}Y and ^{186}Re in patients with hemophilia who were undergoing radionuclide synovectomy (RS) procedure in the last 3 years.
Methods Nineteen patients were enrolled in the study. Most of the patients ($n = 17$) were hemophilia-A (mean age 20.6 ± 10.5 years) and 18 patients (mean age 22.6 ± 10.6 years) with hemophilia who were not exposed to RS procedure were included in the study as control group. Most cases in the control group ($n = 13$) were hemophilia-A. ^{90}Y for knee joints and ^{186}Re for elbow or ankle joints were used to perform RS in hemophilic patients. We studied the micronucleus (MN) test on peripheral blood lymphocytes as an indicator of radiation-induced cytogenetic damage and calculated nuclear division index.
Results There was no significant difference between the patients with and without RS with respect to MN values. However, both values obtained in RS-exposed patients and control group were much elevated than values reported in literature from healthy controls. The mean MN values of patients below 20 years old were much lower but not significant than those above 20 years old. MN frequencies between ^{186}Re and ^{90}Y groups were also analyzed, and no significant difference was observed. Hemophilia patients who were treated with ^{186}Re showed higher levels of MN compared to patients treated with ^{90}Y although the difference was not significant.
Conclusions Radioisotope synovectomy (RS) seems to be a safe procedure not causing a significant genotoxic effect on hemophilic patients, however, further studies including larger series of patients are needed to better understand the effects of RS on patients' health.

Keywords Hemophilia · Radioisotope synovectomy · ^{90}Y (yttrium 90) · ^{186}Re (rhenium 186) · Chromosomal breakages

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Introduction

Radionuclide Synovectomy (RS) is defined as the intra-articular injection of radionuclide agents with the aim of fibrosis on bleeding synovium in the target joint of patients with hemophilia [1–4]. Yttrium-90 (^{90}Y) and rhenium-186 (^{186}Re) are the approved radionuclide agents in Europe. Radioisotopic materials have been successfully used for more than 10 years in target joints for hemophilic children [5, 6].

No oncological transformation has been reported in patients with hemophilia who used radioisotope synovectomy (RS) for more than 30 years. However, 3 years ago, acute leukemia has been reported in two children with hemophilia after RS in the USA [7]. Even though

phosphorus-32 (^{32}P) was the responsible agent in those cases, safety concerns have arisen due to the exposure to all type of radionuclide agents which may cause chromosomal breakages (CBs) and malign transformation.

Genotoxic effects of RS with ^{90}Y and ^{186}Re in hemophilic children have been evaluated using diepoxybutane (DEB) test in a recent study [8] and have not seemed to induce a significant genotoxic effect on peripheral blood lymphocytes. However, chromosomal breakages which were detected even after 1 year of radioisotope synovectomy and de novo chromosomal changes after radioisotopic exposure might be warning signs for young patients. Our previous study showed that RS procedure could be performed after completing the medical options for small children. On the other hand, potential genotoxic effects of RS in patients with hemophilia need to be further studied using more sensitive techniques.

Materials and methods

Patient group

All patients and parents were properly informed before and during the study. Nineteen patients with target joints who performed RS for the last 3 years were enrolled in the study. Most of the patients were hemophilia-A ($n = 17$), and others were hemophilia-B ($n = 2$). All the patients were male. The mean age was 20.6 ± 10.5 years (range 6–52). Only four patients were below 10 years of age. All patients had target joint and/or chronical synovitis before the procedure. Radioisotope synovectomy decision was taken after the evaluation in Hemophilia Council of Ege University Hospital. Radioisotope synovectomy was performed as an outpatient procedure using routine protocol described and published earlier [5, 6]. Two different colloidal radioisotopic agents (^{90}Y and ^{186}Re) were selected for application. In Table 1, physical nature, energy, half-life and capacity of distribution and diameter of colloidals of agents were outlined.

Table 1 Characteristics of radioisotope agents which are used in RS procedure

Radio isotope	Half-life (days)	STP (mm)	β Energy (KeV)	Diameter of colloid (nm)	Preferred joints
^{90}Y	2.7	11	2240	100	Knee
^{186}Re	3.7	3.6	1606	5–10	Elbow–ankle–shoulder
^{32}P	14	7.9	1710	500–2000	All joints

STP soft tissue penetration (maximum)

^{90}Y was used for 11 knees (185 MBq per knee). ^{186}Re was used for 4 elbows and ankles (74 MBq per joint) (CIS Bio International/France). In 4 patients, both radioisotopic agents were used simultaneously in one session. Five patients received more than 2 consecutive sessions of intra-articular injections due to having more than one target joints.

Eighteen patients with hemophilia who were not exposed to RS procedure were taken as control group. Most cases were hemophilia-A ($n = 13$) and others were hemophilia-B ($n = 4$) and von Willebrand disease ($n = 1$). All patients were male. Their age ranged from 5 to 45 years (mean 22.6 ± 10.6).

Micronucleus (MN) assay

The micronucleus test was performed using cytochalasin B (Cyt-B) as described elsewhere [9, 10]. A 0.5 mL of peripheral venous blood was cultured in RPMI medium supplemented with fetal bovine serum, phytohaemagglutinin, penicillin, streptomycin for 72 h. Cyt-B (6 μg/mL) was added at the 44th hour to the blood cultures (final concentration 3 μg mL^{-1}) and incubated for another 18 h and then cultures were harvested. The cultures were centrifuged at 1100 rpm for 10 min. After the supernatant was removed, 10 mL of prewarmed hypotonic solution was added to the pellet and incubated for 23 min at 37°C. The cultures were centrifuged at 1100 rpm for 10 min and the supernatant was removed for 3:1 methanol/acetic acid fixation. Slides were prepared after three fixative changes and leaved for staining with 5% Giemsa for 1 h.

Frequency of mono-, bi- and multinucleated cells, necrotic cells, apoptotic cells were recorded to calculate nuclear division index (NDI). NDI is a measure of the proliferative status of the viable cell fraction and a parameter for comparing the mitogenic response of lymphocytes and cytostatic effects of agents used in the study. A total of 500 viable cells having 1, 2, 3 or 4 nuclei were counted and the NDI was calculated as NDI $= (M_1 + 2M_2 + 3M_3 + 4M_4)/N$, where M_1–M_4 showed the number of cells with 1–4 nuclei and N was the total number of viable cells scored [10]. To prevent commentary differences, 1000 binucleated (BN) cells were scored under the light microscope (400×) by the same researcher. Only cells with well-defined cytoplasmic border and at least 2 nuclei were evaluated for scoring MN in BN cells.

All cytogenetic analyses were performed in the Medical Genetics Laboratories of Ege University Hospital.

Statistical analysis

It was performed using SPSS 13.0 (SPSS Inc., IL, USA) statistical program package. The significance difference for

MN chances was determined by Mann–Whitney *U* and Kruskal–Wallis tests.

Results

There was no significant difference between patients and control group in respect of MN frequencies. However, both values obtained in RS-exposed patients and control group were significantly elevated than values reported in literature from healthy controls [11–14].

Characteristics of hemophilia patients with and without RS are given in Table 2. The distribution of MN frequencies were slightly higher in hemophilia patients but did not differ significantly (Table 3). When all the hemophilia patients were divided into 2 groups according to their age (<20, ≥20), there was no difference; however, the mean value of MN in patients below 20 years old was much lower than those above 20 years old. All patients in the same group were further compared to the control cases who were not exposed to radiosynovectomy. There was no difference between the groups nevertheless the mean MN

Table 2 Characteristics of hemophilia patients with and without RS

	Radioisotope	Patient no.	Patient age	MN (‰)	NDI
Patients ($n = 19$)	^{90}Y	1	23	22	1.10
		2	52	22	2.02
		3	19	12	2.31
		4	18	8	1.08
		5	28	48	1.80
		6	25	24	2.04
		7	19	10	2.12
		8	25	17	1.03
		9	30	24	1.69
		10	9	8	1.51
		11	8	17	2.23
	^{186}Re	12	19	36	2.05
		13	10	20	1.93
		14	17	12	2.43
		15	6	22	2.20
	^{90}Y + ^{186}Re	16	12	7	1.36
		17	27	12	1.10
		18	19	14	1.81
		19	26	12	1.93
Controls ($n = 18$)	None	1	8	11	1.10
		2	19	22	1.16
		3	35	30	1.05
		4	40	8	1.08
		5	5	10	1.09
		6	23	32	2.05
		7	9	8	2.05
		8	45	14	2.21
		9	35	18	2.10
		10	23	20	1.92
		11	21	18	1.56
		12	19	16	2.36
		13	25	8	1.35
		14	19	12	1.28
		15	18	16	1.82
		16	22	22	2.11
		17	23	6	1.02
		18	18	27	2.19

MN micronucleus, *NDI* nuclear division index

value of the patients above 20 years old was much higher than control cases (Table 4). MN frequencies between ^{186}Re and ^{90}Y groups were also analyzed, and although no significant difference was observed, ^{186}Re group had a higher MN frequency than ^{90}Y group (Table 3).

Discussion

Ionizing radiation is a potential danger for humans due to its well-known oncological effects by causing direct DNA breakages [15]. Even though, success rates are very high as more than 80%, no doubt, safety is the first priority in RS especially for children. A few years ago, development of acute leukemia in two hemophiliac children after RS has been reported in the USA by Dunn et al. [7]. The responsible agent in this report was ^{32}P which is the unique approved radioisotope in USA by FDA. Approved radioisotopic agents in Europe are different isotopes such as ^{90}Y and ^{186}Re. ^{32}P has not been approved by European Medicine Agency (EMA) for European countries yet. In our country, due to unavailability of ^{32}P in the market, we have never used this agent for Turkish patients just like other European centers [8].

In fact, ^{90}Y has been used for more than 30 years and no malignancy has been published in hemophilia population until that report. Although some chromosomal aberrations have been occasionally reported after ^{90}Y and ^{186}Re in some patients with rheumatoid arthritis and hemophilia, cytogenetic abnormalities have been observed to return to normal values in the following 1 year follow-up period in those studies. Except the latest publication regarding the relationship between leukemia and radioisotope synovectomy, there are a few publications about radioisotope-mediated malignancy which occurred only in patients with rheumatoid arthritis [16].

Recently, we have studied the effect of RS procedure in patients with hemophilia before and after the therapy [8]. In this study, we used DEB test for the evaluation of chromosomal breakages after RS. CBs were determined in 67.6% of all patients before radioisotope exposure, and after 90 days of exposure, patients who had CBs constituted 61.7% of the study group. However, 3 months after radioisotope exposure, CBs still continued in 21 patients even though the difference compared to the initial values was not found to be significant. Moreover, re-evaluation of 5 patients after 1 year revealed same level of CBs and they were not transient unlike other studies. At conclusion of the previous study, RS with ^{90}Y and ^{186}Re did not seem to induce a significant genotoxic effect on peripheral blood

Table 3 The frequency of MN according to different parameters

	n	MN (‰) Mean	±SD	*p*
Overall				
Patients	19	18.3	10.3	0.680
Controls	18	16.6	7.8	
Age (year)				
<20	11	15.1	8.5	
Patients with RS				
≥20	8	22.6	11.4	0.051
Radioisotope type				
^{186}Re	4	22.5	10.0	
^{90}Y	11	19.3	11.3	0.571

Table 4 The frequency of MN in hemophilia patients with and without RS below and above 20 years old (reference studies were also included)

Age (year)	Studies	*n*	MN (‰) Mean	±SD	*p*
<20	Present study				
	Controls	8	15.3/1000	6.5	0.740
	Patients	11	15.1/1000	8.5	
	Reference study in healthy subjects				
	Pooled analysis: estimated mean values of MN[a]	332	5.7/1000		
	Present study				
	Controls	10	17.6/1000	8.9	0.326
	Patients	8	22.6/1000	11.4	
≥20	Reference studies in healthy subjects				
	Fenech and Morley [9]	42	4.4/500		
	Bolognesi et al. [13]	75	6.67/1000		
	Di Giorgio et al. [11]	200	9.87/1000		

[a] Neri et al. [14]

lymphocytes in hemophilic children. However, as outlined above some patients who still had CBs even after 1 year of observation and de novo chromosomal changes after radioisotopic exposure may be accepted as warning signals for young population.

Turkmen et al. [17] reported a safety study in 20 boys with hemophilia who had undergone RS with ^{186}Re. They used micronucleus assay as in our study to evaluate DNA damage in these patients. No significant difference in the rate of MN has been found between the baseline levels and 90 days after radioisotope exposure. Interestingly, they have outlined that baseline MN count was also significantly increased in patients with hemophilia who were not exposed to RS. We have also found similar results in the control group which had also higher mean value of MN count compared to healthy individuals reported in the literature [10, 12, 14]. The reason why MN count elevated in patients with hemophilia who had not yet been exposed to radioisotope agents has been speculated. There is no single study related to this subject in the literature. In our opinion, the potential reasons may be life-long bleeding episodes in musculo-skeletal tissues and numerous intra-venous supplementation of plasma-derived factor concentrates or blood derivatives such as fresh frozen plasma and cryoprecipitate for treatment of bleedings.

It has been reported that the frequency of MN is increased as the age becomes older which may be called as age effect [12, 14]. This is particularly remarkable above 1-year-old. Major challenges posed by the leaving of the protected intrauterine environment, changes occurring in the first years of age, such as solid diet, vaccinations, and viral diseases could be the likely explanations for this increase [14]. Higher capacity of regeneration following DNA damage in children may be another possible explanation for this difference as well. The micronucleus assay has been reported as one of the best established in vivo cytogenetic assays in the field of genetic toxicology [12, 14, 18]. In our study, the frequency of MN on the patients below 20 years old was lower than those above this age, although the difference is not significant. It is known that as the age increases, there is a decreased DNA repair capacity [19]. Several studies have confirmed that the micronucleus frequency increases with age [20]. There are many studies evaluating the MN frequency in different age groups to show the effect of age [12, 20]. Because there is a limited data on MN frequencies in children [12], the patients are divided into two groups $\geq$20 years and $<$20 years to evaluate the MN frequency difference between children and adults. The age-related decline in DNA repair capacity may have caused those observed differences below and above 20 years old. On the other hand, Rodriguez-Merchan et al. [4] has reported that ^{186}Re produces gamma rays besides beta rays which mean that ^{186}Re has more detrimental effect on human health. In our study, hemophiliac patients who were treated with ^{186}Re showed higher levels of MN compared to patients treated with ^{90}Y which may be due to the characteristics of the radioactive material.

Falcon et al. [16] reported some chromosomal aberrations in 31 patients with hemophilia after RS procedure using ^{90}Y and ^{186}Re. They also pointed out that after 1 year of observation all chromosomal changes were returned to normal. Fernandez-Palazzi et al. [21] also confirmed these results and they have reported that all chromosomal changes were reversible. They also pointed out those changes which appeared similarly in non-radiated patients with hemophilia and disappeared by time. In clinical practice, we have no single patient who has transformed to malignancy after RS procedure throughout 11 years of experience in more than 350 patients with hemophilia. To date, we have reached 11 and 6 years of experience, respectively, using ^{90}Y and ^{186}Re for RS procedure. Both 11 years of experience and in vitro analysis of chromosomal changes after RS procedure in these trials have shown that this procedure, particularly ^{90}Y and ^{186}Re, is safe for young population with hemophilia. On the other hand, concerns related to ^{32}P safety still continue. We speculate that the longest half-life (14 days) capacity of this radioisotopic agent might be related with potential problems.

We have no clinical experience with ^{32}P due to unavailability in the European market. However, further and long-term studies should be performed for better understanding the relationship between leukemia and ^{32}P regarding malign transformation.

Conclusion

According to our studies, RS with use of ^{90}Y and ^{186}Re is a safe procedure for the treatment of chronic synovitis in hemophilic patients who are poorly controlled with medical management.

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