The Capillary Blood *In-Vivo* Micronucleus Test: Wrestlers Exercising at *Akharas*

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Abstract

Some studies have indicated the potential for strenuous exercise to cause genetic damage. In order to investigate whether wrestling can induce any chromosomal damage, micronuclei were scored in small T-lymphocytes of wrestlers using the *in vivo* capillary blood micronucleus (MN) assay. Blood samples from 15 wrestlers and ten controls were analysed. Requisite anamnesic data were collected on a pre-designed proforma. The wrestlers (15– 30y, middle socio- economic class) were mostly non-vegetarians, taking alcohol and smoking. Finger-prick capillary blood samples were processed and 2000 cells per individual were scored for MN. The MN frequency in the wrestlers was statistically significant and increased with age, duration and longer routine of heavy exercising. These observations imply that wrestling causes cytogenetic damage indexed as micronucleated cells resulting from aneugenicity and/or clastogenicity. Wrestlers therefore may be prone to long- term consequence with outcomes in terms of cancer and age-related disease.

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Key Words: Chromosomal damage, strenuous exercise

Introduction

The art of wrestling as a sport and method of defense goes further back in time than any other exercising record of empty-handed combat. It can be traced back to as far as 3400 BC when the Egyptians practiced wrestling as a favourite past-time event. Images of wrestling artifacts can still be seen on the tomb walls in Egypt. As a game, wrestling has been on the maps since as far back as 708 B.C. when it first featured in the Greek Olympics. Gradually, wrestling modified to become a sport with safety guidelines and formal training methods. Amateur wrestling is the most widespread form of sport wrestling. There are two international wrestling styles performed in the Olympic Games under the supervision of FILA (Fédération Internationale des Luttes Associées or International Federation of Associated Wrestling Styles): Freestyle and Greco-Roman. Freestyle is possibly derived from the English Lancashire style. A similar style, commonly called Collegiate, Folkstyle, or Scholastic, is practiced in secondary schools, colleges, and younger age groups in the United States.

Traditional Indian wrestling since 11 AD has however been integral to Hinduism. It is known as *Pahalwani* or *Mallavidya* and is a form of exercise that defines the essence of wrestling and in being human so as to achieve self discipline through physical fitness as well as being an identity and purity of the body, mind and spirit. Trainees reside at the *Akharas* (temples, gyms), leaving civilization and entering the world of tranquility and acknowledgment. *Akharas* are equipped with fine grit dirt floors to bring one closer to natural elements of the earth (sifted and saturated with essential oils to supple the skin of the wrestler when he is wrestling). The oils also keep the dirt clean and compressed for the body to tumble upon.

Natural light and fresh air impact the training area as well to keep it in harmony with the surrounding atmosphere. Exercise is done with one's own body weight. Performing Yoga (posture stretches), *Bethak* (in place squats), *Dand* (push ups), *Jori* (swinging weighted wooden clubs), *Gada* (swinging weighted ball and mace) and body massages give the wrestler a complete regimen. Standardized square earth-filled pits (*Akharas)*, measuring approximately 20'x20'for training and competition were introduced. In India, the authorities have banned the ancient sport of sandpit wrestling in an attempt to help its wrestlers keep pace with the rest of the world by training on mats. Wrestling (or *Dungal*, as often called) has remained a trainers dream and many males opt for it, both as a sport and profession. In *Akharas,* a former wrestler teaches different techniques and tricks of wrestling to the students and also the types of exercises which they have to perform to get fit and ready for the competitions.

Among the effects of exercising is the ability to create free radicals due to increased oxygen uptake. This can cause genetic damage as reported in the literature. DNA damage has been observed in recreationally active individuals, trained athletes and untrained individuals (*Niess et al., 1996; Hartmann et al., 1998; Tsai et al., 2001*). However no literature on genetic damage in tissues of wrestlers has come to attention. Hence the objective of the present study was to score for genetic damage by the capillary blood *in-vivo* micronucleus test in peripheral blood lymphocytes of some wrestlers working out at local *Akharas*.

Materials and Methods

The capillary blood *in vivo* micronucleus test (*Xue et al., 1984*) was performed to score for any chromosomal (micronuclei, MN) damage. Since lymphocytes are distributed throughout the body, circulate in all tissues and are long-lived, therefore cell- cycle kinetics, chromosomal studies, biochemical tests besides others biological investigations, can be carried out on peripheral blood lymphocytes (PBL). The capillary blood *in vivo* micronucleus test scores for micronuclei in the small lymphocytes which have a high nuclear to cytoplasmic ratio. The study involved cytogenetic analysis of 15 male wrestlers and 10 healthy age-matched controls who had never exercised. All the wrestlers (15-30y) were residents of Amritsar city and had been visiting the Shankar *Akhara* and the Kallu *Akhara* for 3-5 years. Male controls in the similar age range were students of the Dayanand Anglo Vedic College, Amritsar. Some wrestlers and controls were non-smokers. Relevant information about the wrestlers and controls was noted on a pre-designed proforma and sample collection was done after their written informed consent. The study was cleared by the Institutional Ethics Committee.

About (150µl) of capillary blood was taken in a heparinized sedimentation tube from a finger-prick made with the help of a lancet. The blood samples were transported to the laboratory and processed for the MN test. The procedure involved adding of methyl cellulose (0.3%) to the heparinized blood sample in a ratio of 1:2 to 1:3; it was mixed carefully with a fine glass rod and the samples were put in a water bath at 37ºC for about 40 min. The lymphocyte suspension was decanted into a micro - centrifuge tube with the help of the micropipette and was centrifuged at 3000rpm for 6 min. The supernatant was decanted except for 0.3ml in which the pellet was re-suspended with the help of

the small vortex mixer. A smear was prepared and the slide was left to air-dry. Fixing of the cells was carried out with 100% methanol for one min. The slides were air-dried, stained in buffered 10% Giemsa (pH-6.4) for 10 min. and mounted in D.P.X. A total of 2000 cells were scored per individual under the low power (40X) of a binocular microscope. The presence of micronuclei in the cells was confirmed at 100X under oil immersion. Slides were randomly scored by another worker to confirm the presence of micronuclei. The criteria for scoring cells for micronuclei were: cytoplasm intact and lying flat, no overlapping between adjacent cells, the nucleus normal and intact, the nuclear perimeter smooth and distinct and only little or no debris . The criteria for identifying micronuclei in the cell were: the size of the micronucleus less than one-third of the diameter of the nucleus, nucleus rounded with smooth perimeter, texture and the staining intensity of micronucleus similar to the nucleus, and no overlapping with the nucleus (*Tolbert et al., 1984*).

Results

Table1. Types of Exercises Performed by Wrestlers

Target Organs	Types of exercise	Daily Duration (min)	Out of n=15, no.of individuals doing various exercises
Chest	Push-ups	20-40	15
Thighs	Sit-ups	20-30	12
Biceps, Triceps	Pull-ups, Rope climbing	40-50	15
Abdominal Muscles	Swinging weighted wooden clubs	30-40	10
Lateral Muscles	Swinging weighted ball and mace	10-20	15

Table 2. General Information about selected males exercising at *Akharas* and control individuals.

Sample Groups	Age (yr.)	Duration of exercising (yr)	Daily exercising (hr)	Warm up ime(min)	Mobile phone	Smoking	Drinking	Veg/non-veg diet	MNd cells/Total cells scored	% MN$ frequency
Wrestlers (n=15)	14-28	2-6	2-6	20-40	8	9	11	13	163/30000	0.54**
Controls (n=10)	17-28	-	-	-	-	3	4	5	16/20000	0.08

$ – Calculated as an average of MNd cells in that group

**Highly significant from controls at 1% level ($p<0.001$;Student's t-test).

The list of various exercises performed by the wrestlers' group is given in Table 1. Genetic damage was observed as MNd cells in 94% of the wrestlers and in 8 % among the control individuals (Table 2). The percentage frequency of MN ranged from 0.50 to 0.65 among wrestlers. A minimum of 2000 cells were scored per sample with minimum of 10 MN and a maximum of 13. The overall frequency of MN in the sample group (0.54 %) was statistically significant when compared with that in (0.08%) control group. The statistical difference for MN frequencies between the wrestlers and the control group reveals that wrestlers are subjected to genetic stress.

Table 3. Chi-Square Test- Comparison of the Sample and Control Group Individuals

Variable	χ^2 calculated	χ^2 tabular 5%	Significant/non Significant	Degree of freedom
Age	1.68	3.14	NS	1
Smoker/Non Smoker	2.95	3.14	NS	1
Alcohol/Non Alcohol	1.82	3.14	NS	1
Mobile User/ Non Mobile User	1.31	3.14	NS	1
Veg/ Non Veg	0.99	3.14	NS	1

Table 4. Regression Coefficient Analysis for Comparison of various Factors with Percent Frequency of MNd Cells

Wrestlers	Age	Alcohol intake	Daily exercise duration	Years since exercising	Mobile Phone usage	Veg/ Non veg	Smoker/Non Smoker	Warming Up
r	0.002	0.010	0.040	0.006	0.054	0.081	0.230	0.040
p	0.946	0.960	0.030	0.040	0.620	0.540	0.530	0.340
Controls								
r	0.005							
p	0.960							

r- Regression coefficient, if (p) = 0.05, test is significant.

Table 5. Multifactorial Analysis of Variance (ANOVA) of Independent Variables for MNT: Persons Doing Heavy Exercise in *Akharas* and of Control Individuals

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F-ratio	Significant (p-value)
Age	154.500	12	16.564	0.874	NS
Alc. Intake	2.984	12	0.876	0.956	NS
Daily Duration	3.430	12	0.654	0.564	S
Time Since exercising	30.640	12	2.453	3.548	S
Mobile Phone usage	2.540	12	2.432	9.876	S
Veg/Non veg	3.560	12	9.756	1.987	NS
Smoker/Non Smoker	3.654	12	4.873	4.634	NS
Warming up	654.870	12	5.342	1.342	S
Control					
Age	123.650	12	9.654	0.321	NS

Table 6. Effect of Age on Chromosomal Damage in Wrestlers.

Age (y)	No.	Age (y)		Time since exercising (y)		Daily exercise (hr)		Warm-up (min)		MNed cells/Total cells scored	% MN$ frequency±SEM
		Range	Mean	Range	Mean	Range	Mean	Range	Mean		
Wrestlers											
14-20	5	14-19	17.0	2-6	3.4	3-4	3.6	20-40	29.0	57/10000	0.41**±0.081
20-25	6	22-23	22.5	2-6	4.0	2-4	4.5	20-40	29.1	63/12000	0.37*± 0.089
25-30	4	25-28	26.0	3-6	4.5	3-6	4.2	20-40	30.0	43/8000	0.48**± 0.067
Controls											
14-20	4	16-19	17.5							6/8000	0.05± 0.001
20-25	3	20-23	21.6							4/6000	0.06± 0.009
25-30	3	25-28	26.6							6/6000	0.06± 0.009

$ – Calculated as an average of MNd cells in that group, *Significant from parallel controls at 5% level (p<0.05; Student's t-test) and **Highly significant from parallel controls at 1% level p<0.001; Student's t-test). Non-significant within wrestlers' group.

Table 7. Effect of Years of Exercising on Chromosomal Damage in Wrestlers.

Time since Exercising (yr)	No.	Mobile Phone users	Smokers	Alcohol Intake	Age Range (y)		Daily exercise (hr)		Warm-up (min)		MNed cells/Total cells scored	% MN$ frequency±SEM
					Range	Mean	Range	Mean	Range	Mean		
Wrestlers												
1-3	6	4	3	3	14-25	19.6	2-4	3.5	20-30	22.5	65/12000	0.43**±0.068
4-6	9	4	6	8	18-28	22.8	2-6	3.5	25-40	33.8	98/18000	0.54**±0.076
Controls												
	10	3	4	2	15-30	24.0					16/20000	0.06±0.008

$ – Calculated as an average of MNd cells in that group. **Highly significant with total control at 1% level (p<0.001; Student's t-test). Non-significant within wrestlers' group.

Table 8. Effect of Daily Exercise on Chromosomal Damage in Wrestlers.

Daily Exercise (hrs)	No.	Mobile Phone users	Smokers	Alcohol Intake	Age Range (y)		Years since exercising (y)		Warm-up (min)		MNed cells/Total cells scored	% MN$ frequency±SEM
					Range	Mean	Range	Mean	Range	Mean		
Wrestlers												
1-2	2	0	1	1	23	23.0	3-4	3.5	20-40	30.0	23/4000	0.57**±0.075
3-4	12	8	7	9	18-28	21.08	2-6	3.8	20-40	29.5	120/24000	0.51**±0.081
5-6	1	0	1	1	25	25.0	6.0	6.0	25	25.0	10/2000	0.43**±0.061
Controls												
	10	3	4	2							16/20000	0.08±0.008

$ – Calculated as an average of MNd cells in that group. **Highly significant with total control at 1% level (p<0.001; Student's t-test). Non-significant within wrestlers' group.

Table 9. Effect of Warm-Ups on Chromosomal Damage in Wrestlers.

Warm-up (min)	No.	Mobile Phone users	Smokers	Alcohol Intake	Age Range (y)		Daily exercise (hr)		Time since exercising(y)		MNed cells/Total cells scored	% MN$ frequency±SEM
					Range	Mean	Range	Mean	Range	Mean		
Wrestlers												
20-30	7	5	5	5	14-25	21.1	2-6	3	2-6	3.2	73/14000	0.38**±0.081
30-40	5	2	2	4	18-26	21.4	3-4	3.2	2-6	4.2	54/10000	0.31**±0.071
40-50	3	1	2	2	18-28	23.0	2-4	3.3	4-6	5.0	36/6000	0.43**±0.066
Controls												
	10				16-28	22.5					16/20000	0.07±0.009

$ – Calculated as an average of MNd cells in that group. **Highly significant with control group at 1% level p<0.001;Student's t-test). Non-significant within wrestlers' group

Table 10. Effect Of Height on Chromosomal Damage in Wrestlers.

Height (cm)	No.	Mobile Phone users	Smoker	Alcohol Intake	Age Range (y)		Daily exercise (min)		Time since exercising (y)		Warm-up (min)		MNed cells/Total cells scored	% MN$ frequency±SEM
					Range	Mean	Range	Mean	Range	Mean	Range	Mean		
Wrestlers														
160-170	5	2	2	3	18-28	22.6	2-4	3.4	2-6	4.2	30-40	37	59/10000	0.48**±0.069
170-180	4	3	2	3	14-23	18.75	2-4	3.2	2-3	2.2	20-25	21.2	41/8000	0.52**[b]±0.081
180-190	6	3	5	5	19-25	22.6	3-6	3.8	3-6	4.8	20-35	28.3	63/12000	0.42*[c]±-0.068
Control														
160-170	6	2	3	2	16-28	22.5							10/12000	0.08±0.009
170-180	2	1	1	0	17-22	19.5							3/4000	0.06±0.008
180-190	2	0	0	0	18-23	20.5							3/4000	0.05±0.007

$ – Calculated as an average of MNd cells in that group, *Significant from parallel controls at 5% level (p<0.05;Student's t-test) and **highly significant within groups at 1% level p<0.001;Student's t-test) except [b,c]

Table 11. Effect of Weight on Chromosomal Damage in Wrestlers

Weight (kg)	No.	Mobile Phone users	Smoker	Alcohol Intake	Age Range (y)		Daily exercise (min)		Time since exercising (y)		Warm-up (min)		MNed cells/Total cells scored	% MN$ frequency±SEM
					Range	Mean	Range	Mean	Range	Mean	Range	Mean		
Wrestlers														
60-70	4	2	4	4	19-28	23.0	3-4	3.5	4-5	4.7	25-40	32.5	42/8000	0.31±0.078
70-80	5	2	1	4	18-26	22.2	2-4	3.2	2-6	3.4	20-35	29.0	53/10000	0.41±0.086
80-90	3	1	3	2	14-25	20.6	2-6	3.6	2-6	4.0	20-40	28.3	33/6000	0.38±0.075
90-100	3	3	1	1	16-25	19.6	4-4	4	2-6	3.6	20-40	26.6	35/6000	0.41*±0.067
Control														
60-70	3	1	1	1	18-23	21.0							3/6000	0.05±0.007
70-80	2	0	1	0	19-20	19.5							4/4000	0.08±0.001
80-90	4	2	1	1	16-28	21.5							7/8000	0.07±0.009
90-100	1	0	1	0	27	27.0							2/2000	0.05±0.010

$ – Calculated as an average of MNd cells in that group, *Significant from parallel control at 5% level (Student's t-test). Non-significant within and between other groups.

The 2x2 contingency Chi-square (χ^2) test was performed to find out if the control group matched the sample group with respect to demographic, dietary and life style features (Table 3). The groups did not differ and hence matched. Multiple regression analysis was performed to assess the possible relationship of various independent variables which could be confounding factors i.e. age, weight, alcohol consumption, use of mobile phone, dietary pattern (non-vegetarian) in physically active and control individuals for micronuclei induction (dependent variable). The calculated correlation coefficient 'r' and probability 'p' values revealed that daily exercise duration and years of exercising contributed to chromosomal damage (Table 4). The analysis of variance (ANOVA) revealed that significant increase in MN induction was observed for daily exercise time, time-since-exercising, mobile phone usage and for warming-up (Table 5).

The Student's t-test was performed to analyse whether age, weight, height, time-since-exercising, daily exercise time, warm-up time exhibited genetic damage. The distribution of percentage frequencies of MNd cells in various age groups of both wrestlers and control group is presented in Table 6. The mean time of exercising of the wrestlers is 5-6 years. The MN frequency in the youngest age range was between 0.57 to 0.07 in the wrestlers with the mean time of exercising of 3.4 years while it was highest (0.53 to 0.10) in the 25-30 years interval with a mean time of exercising of 4.5 years. Significantly elevated frequency of MNd cells was observed between the damage in wrestlers' and parallel controls but this was non-significant within wrestlers' group. For effect of time-of-exercising (Table 7), daily duration (Table 8) and warm-up (Table 9) on chromosomal damage highly significant differences were observed with total control at 1% level but none within the wrestlers' group. Significant differences from parallel controls were also observed for height (Table 10) but not for weight (Table 11) for which significance from parallel control was seen in the most heavy category.

Discussion

Endurance exercise elicits a 10-20 folds increase in whole body oxygen (O_2) consumption which at the level of the skeletal muscle increases 100-200 folds. This increase in O_2 utilization may result in the production of reactive oxygen species (ROS) at rates that exceed the body's capacity to detoxify them (Alessio, 1993). Left unchecked, these ROS may cause protein, lipid, and/or DNA damage. Numerous studies have shown that excessive exercise can result in oxygen radical-mediated injury and various biochemical mechanisms of free-radical generation after strenuous exercise have been identified (*Hartmann et al., 1998*). Besides adverse effects on muscle tissue, exhaustive exercise is also capable of causing temporary immunomodulations such as changes in the populations and/or in the activities of immunocompetent cells. Rather, heavy exertion in contrast to moderate exercise has adverse effects on the immune system as well as on DNA damage leading to the formation of micronuclei which can lead to cancer (*Umegaki et al., 1998*).

The observations from the present study also indicate the genetic damaging effect from wrestling. In the literature available, various studies have also reported damage to the genetic material after exhaustive exercises. Sporting activities like treadmill running (*Niess et al., 1996*) and other endurance exercises also resulted in DNA damage (*Mastaloudis et al., 2004*). Exercise-induced DNA damage also has been reported in recreationally active individuals (*Hartmann et al., 1995; Mars et al., 1998; Niess et al., 1998*), as well as in trained (*Niess et al., 1996*) and untrained athletes (*Niess et al., 1996; Schiffl et al., 1997; Hartmann et al., 1998; Tsai et al., 2001*).

In the light of results of the present study and the literature reviewed, there is clear evidence that strenuous exercise for strength training (wrestling) can lead to chromosomal damage. Individuals training for such activities vignettes cautions in view of the consequences like cancer and disease resulting from genetic damage.

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