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Research Paper

GENETIC INJURY STUDIES IN JUDO PLAYERS

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Objective- To assess genomic (DNA and chromosomal) damage in judo players. **Design-** The study was approved by Institutional Ethics Committee. The players for the present study were contacted from the university during their training. **Setting-** The blood and buccal samples were collected in the morning session before the warm-up and the start of their training session. For DNA and chromosomal damage assessment, the Single Cell Gel Electrophoresis (SCGE) and buccal micronucleus (MN) cytome assays were performed respectively. Participants- Volunteers (n=27) included judo players competing at inter-university and state levels and age- and sex-matched healthy controls. **Results-** Highly significant DNA damage in blood leucocytes as well as chromosomal damage (cytokinetic defect and cell death) in buccal epithelial cells of Judo players was observed. No gender differences were found. Significant associations were observed between some genetic damage parameters and sport related variables (warm-up time and time/years of exercising). **Conclusion-** The genetic injury/damage in different cells of the players requires intervention and management gives the tendency for it to cause malignancy, age related diseases and neurological disorders.

Keywords: Genetic injury, DNA damage, Chromosomal damage

INTRODUCTION

Judo, as a combat sport and an ideal form of physical exercise is a high intensity sport in which anaerobic and aerobic systems are involved to meet the physiological demand requiring competitive endurance (Koury et al., 2005). It is classified as a high static, low dynamic sport (Mitchell et al., 2005) demanding great strength being characterized by high intensity intermittent exercises in preparation for the required explosive effort (Radovanovic et al., 2009). However, although physical training tends to enhance the anti-oxidant defense system (Clarkson and Thompson, 2000; Amani et al., 2010) yet repeated bouts of exercise causes increased generation of various forms of free oxygen radicals (Finaud et al., 2006; Berzosa et al., 2011). This excessive generation of reactive oxygen species has a harmful tendency as they can cause oxidative modifications of lipids, proteins and DNA (Khansari et al., 2009; Packer et al., 2008). Excessive strenuous exercising and sports'

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activities with concurrent generation of free radicals and oxygen- mediated injury have been reported to cause DNA damage (Mastaloudis et al., 2004; Orhan et al.,2004; Tsai et al., 2001; Hartmann et al., 1999). However, combative sport has been sparsely investigated for genetic injury. Rather to the best of our knowledge, no study is available on genetic damage in Judo players (Judokas). Hence in the present study, both DNA damage in peripheral blood leucocytes and chromosomal damage in buccal epithelial cells were investigated in judo players. The significance of genetic injury/damage is in its manifestation as pre-cocious age-related changes, neurological diseases and in malignancy (Halliwell et al., 2000; Marnett, 2000). Given the popularity of the sport as a form of defense and the ready availability of this sports' option in schools and colleges, the investigations for genetic injury gains importance since an early detection of such damage can assist in formulation of preventive and management strategies. The Single Cell Gel Electrophoresis (SCGE/Comet) and the buccal cytome assay are widely used and well validated techniques for genetic damage investigations. The alkaline SCGE assay has various applications including for human bio monitoring (Singh et al., 1988) and the buccal micronucleus (MN) cytome assay also finds favour because of its minimally invasive approach for cytogenetic surveillance (Thomas et al., 2009).

Damage to DNA studied by the SCGE assay includes single- and double- strand DNA breaks, alkali labile sites, cross-links and is mostly assessed in peripheral blood leucocytes (PBL) as they are long-lived and easy to obtain and therefore are ideal for studying genetic damage (Kopjar et al., 2002). The assay enables both quantitative estimation of genetic injury in terms of number of comets for calculation of damage index (DI), damage frequency (DF) and extent of DNA damage (DNA migration length). Chromosome damage as clastogenic (chromosome breaking) and/or aneugenic (chromosomal non-disjunction) events is observable using the buccal MN cytome assay in buccal epithelial cells as it is used as an indicator of genotoxic exposition. This assay reflects the genotoxic events that occurred in the dividing basal layer, one-to-three weeks earlier (Diler and Celik, 2011). Micronuclei (MN) are whole chromosomes or acentric chromosome fragments which fail to incorporate into the main nucleus on cell division but form a small nucleus separately. Keeping the sensitivities of the assays in assessing genetic damage, these well-validated techniques were employed to study genetic injury in cells of different tissues of some Judo players.

MATERIALS AND METHODS

Subject Selection: The study included healthy Judo players and controls matched for age, sex and socio-economic status. Male (playing under 80kg weight category) and female (playing under 63 kg weight) judokas comprised the study group. Healthy individuals not exposed to any toxicants at work or incidentally, comprised the control group.

Inclusion Criteria: Judokas competing at inter-university and state levels and in this sport for at least three years with a daily physical training programme of three hours for improving specific skills (randori and drills) as required for the sport.

Exclusion Criteria: Players/ participants on medication or on drugs or supplements during the last six months.

Study Design: The study was approved by Institutional Ethics Committee. Judo players were contacted from university gymnasium. After voluntary written informed consent, a face to face interview was held with each subject and their demographic details, pedigree charts, sport-related/occupation-related information and life style patterns were recorded on a specially-designed proforma.

Anthropometric Measurements: These were taken as per standard protocol (Weiner and Lourie, 1981). Records were maintained for height (cm), weight (kg), hip circumference (cm), waist circumference (cm). From the measurements obtained, Body Mass Index (BMI) and Waist-Hip Ratio (WHR) were calculated to assess the subjects for obesity since this condition has increased manifolds across all population strata.

Sample Collection (Peripheral blood leucocytes and buccal epithelial cells): After explaining the details of the study to the participants, blood and buccal samples were collected during the morning session before the warm-up and the start of their training session.

Injury to genetic material was assessed at both DNA and chromosomal levels simultaneously in two tissues viz, the buccal epithelial cell population and the peripheral blood leucocytes (PBL) respectively. The significance of genetic assessment of these two tissues lies in the fact that both repaired (chromosomal) and unrepaired (DNA) damage can be evaluated in minimally invasive manner.

SCGE/ Comet Assay: For DNA damage assessment, the alkaline SCGE assay was performed using fresh blood samples as per the method of Singh et al (1988) except for use of locally available chemicals, agarose-coated slides and silver staining of comets. The principle behind this method is that there occurs migration of DNA fragments (if there is DNA damage) from the nucleoid in an electric field and is observed in the form of a comet shape (Dusinská and Collins, 2008).

For the assay, blood sample (25µl) was mixed with low melting point agarose (75µl, 0.5% LMPA) and layered onto a pre-coated (100µl of 1% normal melting point agarose) glass slide, followed by third layering onto LMPA. The slide preparation was then kept in chilled, freshly prepared lysing solution (2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris, pH 10) for 2-3h. The slides were then immersed in freshly prepared electrophoretic buffer (pH>13 at 4ºC) consisting of 300mM NaOH, 1mM EDTA for DNA unwinding. An electrophoretic run was conducted for 30min (25V/300mA) and after electrophoresis; the slides were neutralized with 0.4 M Tris and stained with silver nitrate. The coded slides were then screened under a binocular light microscope at 40X and 100 cells were scored per sample (50 cells per slide). Each cell was categorized according to damage to the nucleoid (Collins, 2004) graded from 0-4 (undamaged to maximally damage). Damage index and Damage frequency were obtained as given by Franke et al., (2005).

Damage index was calculated by summing undamaged cells (category 0), number of cells in category I multiplied with one, number of cells in category II multiplied with two + those in category III multiplied with three, etc. Damage frequency (DF) was obtained by adding cells with tails in all categories DNA migration length (µm) was measured at 40X using an ocular micrometer fitted into the eye piece of a transmission binocular microscope. The difference between the length of the comet and the radius of the nucleoid was the extent of DNA damage at cell level.

The Buccal Micronuclei (MN) Cytome Assay: For obtaining buccal epithelial cells, the participants were asked to rinse their mouth thoroughly with water to remove debris. Epithelial scraps from both cheeks were collected on separate slides were prepared, air dried for 10 min and then fixed in Carnoy's fixative [3 methanol: 1 acetic acid]. Slide preparation were then hydrolyzed (1N HCl; 8-10 min) followed by staining with Aceto-orecin (2%) and counter-staining with Fast Green (1%). This causes the MN and nuclei to stain green and the cytoplasm is stained pink (Stich et al., 1982). The slides were again air dried, coded and scanned under a binocular light microscope at 40X. A total of 2000 cells per individual were assessed for micronuclei. Initially 500 cells per slide for DNA damage (nuclear buds), cell proliferation (basal cells), cytokinetic defects (binucleated cells) and cell death (condensed chromatin, karyorrhexis, pyknotic and karyolitic cells) as per Tolbert et al., (1992) scored. The criteria followed for screening MN and micronucleated cells. Random verification of MN was done by another scorer. Repair index (RI) was calculated using the formula: RI=KR+KL/BE+MN (also cells with karyolysis +karyorrhexis/ broken egg + micronucleated cells). The RI is a measure of genotoxicity of cancer. As in the case of MN, nuclear buds (or BE) are also considered as the first morphological stage and are related to genotoxicity in buccal epithelial cells (Diler and Celik, 2011).

Statistical Analysis: The values for each of the genetic parameters scored were taken as mean± SEM. The significance for comparisons of all parameters between control and players were analyzed using Student's t-test. The analysis of variance (ANOVA), Pearson correlation and linear regression were performed to find association and linear relationship (if any) of the studied parameters with the confounding factors. Chi-square analysis was also carried out to find any significance of different cell categories within the study group and with the controls. In order to verify if players and controls matched, Chi-square test was conducted on values of their physical attributes. The SPSS version 16 was used for the analysis and values were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The observations from the present study highlights that judokas assessed for genetic injury in cells of two different tissues have significantly higher levels of DNA and chromosomal damage compared to values in healthy controls not performing any strenuous physical activity. The demographic characteristics of the judokas and control individuals gender-wise are presented in Table 1. The study group included 17 judokas (4 Males, 13 Females) playing judo for at least three years with a daily warm-up time of 15-30 min. The age- and sex matched individuals (n=10, 4 Males and 6 Females) without any involvement in any type of sport participated as healthy controls. The participants were all students (18-25y) non-smoking, not taking alcohol or any food supplementation. The groups matched each other for their physical attributes and life style except for mobile phone usage. The descriptive statistics (combined for genders) are presented in Table 2.

In Table 3 is presented the analyses (Student's t-test) of DNA (unrepaired) and chromosome (repaired) damage on comparison with genetic damage assessed in controls. All the genetic instability parameters were significantly elevated ($p<0.000$). Genetic damage in male and female players were not significantly different. Similarly no

Table 1: General Information of Judo Players and Controls Individuals-Gender Wise

Characteristics			Number of Sports Individuals n=17 (%)			Number of Controls Individuals n=10 (%)			Chi–square Test	
			Male	Female	Total	Male	Female	Total	χ^2	
Age (y)	18–25		04 (23.53)	13(76.47)	17	04 (40)	06(60)	10	–	–
Height (cm)	145–167.99		–	13(76.47)	13	01(10.00)	06(60)	07	0.137	NS
	168–190.99		04(23.53)	–	04	03(30.00)	–	03		
Weight (kg)	40.0–65.0		01(5.88)	12(70.59)	13	02	06	08	0.045	NS
	66.0–90.0		03(17.65)	01(5.88)	04	02	–	02		
Time–since–playing (y)	≥3 –7y		01 (5.88)	09 (52.94)	11	–	–	–	–	–
	≥7–12y		03 (17.65)	04 (23.53)	06	–	–	–	–	–
Warm–up (min)	15–30		04	13	17	–	–	–	–	–
BMI Range* (kg/m^2)	Normal 18–22.9		02	11	13	03	05	08	0.045	NS
	Overweight 23–24.9		02	02	04	01	01	02		
Life Style/Habit	Dietary	Non–veg	03	05	08	02	02	04	0.0105	NS
	habit	Veg	01	08	09	02	04	06		
	Mobile	Yes	04	06	10	04	06	10	5.56	S
	phone usage	No	–	07	07	–	–	–		

**Misra et al., 2009*

Table 2: Demographic Information (Combined for Genders) of the Judo Players and Control Individuals

Characteristics	Judo players (n=17)	Controls (n=10)
Age (y)	19.82±0.35	20.10±0.43
Height (cm)	163.3±2.58	163.06±3.29
Weight (kg)	58.89±2.46	58.87±2.35
BMI (kg/m^2)	21.99±0.39	22.10±0.26
Waist circumference (cm)	30.31±0.88	29.25±0.55
Hip circumference (cm)	34.67±0.75	33.25±0.55
WHR	0.87±0.01	0.88±0.008
Time Since Exercising (y)	6.76±0.57	–
Warm-up (min)	20.58±1.76	–

gender differences for genetic damage were observed in controls. DNA damage in PBL (comet assay) was significantly elevated in players. In terms of cells showing damage (percent cells with tails), there were ~2.5 times more cells with damage in judokas being highly significant ($p<0.001$). The extent of DNA migration was similarly highly significant ($p<0.001$) in players as was the DI, the latter being ~2.5times higher ($p<0.001$).

Table 3: DNA Damage and Chromosome Damage in Judo Players and Controls

Genetic Damage	CONTROLS			PLAYERS		
	Range		Mean±S.E.M.	Range		Mean±S.E.M.
	Minimum	Maximum		Minimum	Maximum	
The SCGE Assay for DNA damage						
Percent cells with Tails (DF)	17.00	65.00	35.00±4.33	74.00	100.00	93.94***±2.21
Mean DNA migration length (μm)	14.02	22.71	18.29±0.78	18.48	42.69	26.67***±1.62
Damage Index (DI)	20.00	80.00	43.90±5.48	79.00	161.00	107.92***±4.21
The Buccal MN Cytome Assay for Chromosome damage						
Micro nucleated (MN) cells	0.05	0.30	0.18±0.03	0.20	0.65	0.35***±0.20
Nuclear buds (NBuds)	0.15	0.40	0.30±0.026	0.70	1.25	0.96***±0.05
Basal cells (BC)	0.50	0.40	0.65±0.04	1.50	3.30	2.52***±0.12
Binucleated cells (BNC)	0.30	0.70	0.55±0.037	0.90	2.30	1.59***±0.09
Karyorrehectic cells	1.50	2.70	1.95±0.16	2.30	3.50	2.92***±0.09
Condensed chromatin	1.20	2.20	1.63±0.11	21.00	35.00	27.06***±1.12
Karyolitic cells	3.10	5.30	4.70±0.19	4.70	7.10	6.43***±0.13
Pyknotic cells	0.00	0.30	0.14±0.03	0.20	0.70	0.47***±0.04
Repair index (RI)	4.00	18.75	7.58***±1.34	2.33	4.42	3.40±0.16

Note: ***Highly significant ($p<0.001$) when compared to control group (Student's t-test); ***Highly significant ($p<0.001$) when compared to subject group (Student's t-test).

Chromosome damage measured as cells with MN in buccal epithelial was twice as high and significantly elevated ($p<0.001$) in players. The cytome parameters: Cell proliferation (as measured as number of basal cells), cytokinetic defects (binucleated cells), cell death (cells with condensed chromatin, karyolytic, karyorrhectic, pyknotic cells) and DNA amplification/damage (nuclear buds) were significantly ($p<0.001$) increased in players. The repair index however was significantly decreased in players indicating that apoptosis (cell death) was increased at the exposure of genomic damage.

In order to assess whether extent of DNA damage (comet tail) graded and categories 0-III was significant in players and controls (Table 4), χ^2 analysis revealed significantly highest category I cells in players followed by those of category II. These cell categories were significantly lower in controls while category III was absent altogether.

Factors known to increase genetic damage (confounding factors, sports related variables) were subjected to analyses (ANOVA, Pearson correlation and Linear Regression) to find out whether these were contributors to genetic injury (Table 5). Among confounders (age, dietary pattern and mobile phone usage), age had a significant association with damage index ($p=0.027$) and mean DNA migration ($p=0.006$) and diet with mean DNA migration ($p=0.001$) and

pyknotic cells (p=0.019). Use of mobile phone shared association with mean DNA migration (p=0.002), binucleated (p=0.05) and pyknotic (p=0.047) cells. In case of sports related variables, warm-up time showed association with DNA migration (p=0.001), binucleated (p=0.022), karyorrhectic (p=0.049) and pyknotic (p=0.022) cells. Time since exercising was also associated with DI (p=0.05), BNC (p=0.003) and karyorrhectic (p=0.013) cells. In controls, only dietary pattern shared association (p=0.052) with DI. The same level of significance was performed when Pearson correlation and regression analysis were performed.

Pearson correlation analysis also revealed negative correlation of RI with MN cells (p=0.041, r= -0.500) and of RI with NBuds (p=0.026, r= -0.517) i.e. with increase in chromosome and DNA damage in Table 6, there was more cytotoxicity in the buccal epithelium cells.

In the present study, increased levels of genetic damage of DNA and chromosomal levels were observed in judokas when compared to levels in controls though no gender differences were observed. Sport-related variables (warm-up time and time since exercising) showed significant associations with genetic damage indices. The observable damage to the genetic material both unrepaired and after DNA repair, may have arisen as secondary effects resulting from strenuous and combative exercises being performed by the judokas. Though to the best of our knowledge this is for the first time that genetic damage has been assessed in judo players, yet regular exercising and other sports' activities have been reported to lead to DNA (Gandhi and Gunjan, 2009; Mastaloudis et al., 2001) and chromosomal damage(Gandhi et al., 2008; Gandhi and Mahajan, 2007; Gandhi and Kumar, 2007).

Reinforcement of physical strength and skill in competitive sports and setting of physical load levels in training camps for players caused some significant changes at cellular or humoral levels in the functioning of the immune system (Yamai et al., 2011). In fact, even though the mechanisms inducing exercise-induced DNA modifications in relation to immune and inflammatory responses to prolonged intense physical activity is not yet well understood (Marini and Veicsteinas, 2010; Neubauer et al., 2009), the dysfunctional role of reactive oxygen and nitrogen species (generated during exercise-training) in causing oxidation of

Table 4: Cells with Different Grades of DNA Damage of Judo Players and Control Individuals

Groups		Grades of DNA Damage			
		Category 0	Category I	Category II	Category III
Judo	Male	33	346	21	–
	Female	70	1040	144	35
	Total	103	1386	165	35
Control	Male	228	134	39	–
	Female	422	139	39	–
	Total	650	273	78	–
χ^2		0.241 (p=0.62)	**63.33** (p<0.001)	**37.59** (p<0.001)	–

Note: Values in bold are significant (Chi-square test)

Table 5: Multivariate ANOVA, Pearson Correlation and Multiple Linear Regression Analysis of Comet Parameters in Judo Players and Controls

		Age	Diet	Mobile	Warm-up	Time Since Exercising
Judokas						
Damage index (DI)	Mean square	1385.682 229.590	465.413 290.941	512.572 287.797	1124.051 247.032	503.698 288.389
	Beta coefficient	-0.536	0.310	0.326	0.323	-0.482
	r-value	-0.536	0.310	0.326	0.323	-0.482
	p-value	**0.027**	0.225	0.202	0.206	**0.050**
Mean DNA length migration (μm)	Mean square	287.973 28.404	372.209 22.789	354.881 24.000	377.881 22.410	148.263 37.718
	Beta coefficient	-0.635	0.722	0.704	0.727	-0.456
	r-value	-0.635	0.722	0.704	0.727	-0.456
	p-value	**0.006**	**0.001**	**0.002**	**0.001**	0.066
Binucleated cells	Mean square	0.059 0.145	0.325 0.127	0.517 0.114	0.679 0.103	1.010 0.081
	Beta coefficient	0.162	-0.362	-0.481	-0.552	0.673
	r-value	0.162	-0.362	-0.481	-0.552	0.673
	p-value	0.533	0.131	**0.05**	**0.022**	**0.003**
Karyorrhectic cells	Mean square	0.015 0.167	0.311 0.147	0.551 0.131	0.589 0.128	0.873 0.109
	Beta coefficient	0.076	-0.351	-0.468	-0.484	0.589
	r-value	0.076	-0.351	-0.468	-0.484	0.589
	p-value	0.771	0.167	0.058	**0.049**	**0.013**
Pyknotic cells	Mean square	0.000 0.025	0.119 0.017	0.089 0.019	0.114 0.017	0.010 0.024
	Beta coefficient	-0.025	0.562	0.487	0.550	-0.161
	r-value	-0.025	0.562	0.487	0.550	-0.161
	p-value	0.923	**0.019**	**0.047**	**0.022**	0.538
Controls						
Damage index	Mean square	448.90 282.00	1065.376 204.940	589.067 264.479	–	–
	Beta coefficient	0.407	0.628	0.467	–	–
	r-value	0.407	0.628	0.467	–	–
	p-value	0.243	**0.052**	0.174	–	–

Note: Values in bold are significant

nucleic acids is clearly well documented (Khansari et al., 2009; Sachdev and Davies, 2008). The generation of free radicals occurs during normal physiological conditions and excessively formed are controlled by cellular defence mechanisms (Rojas et al., 2011). In the course of doing exhaustive/ severe or moderate exercises, this production of free radicals is increased tremendously because of uptake of oxygen resulting in a state of oxidative stress if

Table 6: Pearson's Correlation of Buccal Cytome Assay Parameters with Repair Index in Judo Players

Pearson's Correlation		Micronucleated cells	Nuclear buds	Karyolitic cells
Repair index (RI)	Mean square	0.120 0.024	0.158 0.026	1.293 0.236
	Beta coefficient	-0.500	-0.538	0.517
	r-value	-0.500	-0.538	0.517
	p-value	**0.041**	**0.026**	**0.034**

Note: Values in bold are significant

antioxidants, are less or decreased (Bonilla et al., 2005). The oxidative stress has ramifications in terms of oxidation of cellular components viz. lipids, proteins and nucleic acids. Repeated exercising caused damage to DNA (Okamura et al., 1997). After intensive endurance exercise, the reactive oxygen species released from neutrophils damaged leukocytic DNA (Tsai et al., 2001; Niess et al., 1998).

Oxidative damage to genetic material was reported to lead to DNA strand breaks (Pitozzi et al., 2003; Liu et al., 2002) and micronuclei (Chen et al., 2006). Carcinogenic consequences of oxidative DNA damage and their association with chromosomal lesions (MN) are well documented (Bonassi et al., 2007; Gomez-Meda et al., 2004).

Muira et al. (2005) reported a significant increase in reactive oxygen species (ROS) production in judokas performing daily acute exercise loads. The present study could not measure ROS but significantly elevated DNA and chromosomal damage was observed in two different cell types of judokas when compared to values in matched controls with no sport activity. The MN observed in epithelial buccal cells was formed in the basal layer of the epithelial tissue. After the normally occurring rapid turnover of epithelial tissue, these cells containing the MN migrated to the surface of the epithelium (Rebiero et al., 2008; Torres-Bugarin et al., 2007) and has chromosomal lesions hence are genetic damage remmanants after DNA repair.

The DNA damage assessed in PBL is unrepaired genetic damage since leucocytes are long-lived differentiated cells which do not divide in vivo and hence any DNA damage in them cannot have been subjected to repair.

The increased oxidative stress and inflammatory responses triggered by the regions physical demands of Judo could in all probability have caused genetic injury in the different tissues which persisted both after subjected to repair in buccal cells (MN) and unrepaired (DNA damage) in PBL. As such genetic lesions are early indicators of neoplasia and can lead to neurological and age-related diseases (Halliwell, 2000; Marnett, 2000). Judokas assessed in the present study may be at increased risk from such consequences.

However, the limitation of the present study in terms of small sample size and the inability to estimate oxidative stress biomarkers due to constraints in sample availability/ donation need to be redressed and results confirmed in a larger group.

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