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The effect of strength training on oxidative DNA damage in the elderly

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Meinen Eltern und meinem Lebenspartner gewidmet

Affirmation

Hereby I declare that the master thesis submitted was in all parts exclusively fabricated on my own and that other resources or other means, than those explicitly referred to have not been utilized. All passages quoted from publications or paraphrased from these sources are properly cited and marked as such. The work was not submitted previously in same or similar form to another examination committee and was not yet published.

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Irina Demund

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1. INTRODUCTION

The population in Europe is maturing expeditiously. The mean age of Europeans is the highest in the world (WHO, 2012). The percentage rate of elderly ≥60 years is rising more rapidly than any other population group in Europe. In many European countries the life expectancy already exceeds 75 years for men and 80 years for women (WHO, 2012). It is expected that the proportion of elderly ≥60 years will increase up to 1.2 billion in 2025 and 2 billion in 2050 in the whole world (WHO, Ageing and Life course 2012). In this context it is important to develop suitable programs and guidelines for healthy aging. Nowadays and in near future aging will be an important issue for politics, society and research. We will have to deal with problems like: chronic diseases, dementia, high costs of health care, and mortality. Sedentary life style is also a problem in aging people. It is accompanied with a decline in skeletal muscle mass and loss of muscle strength, resulting in sarcopenia. Common problems of sarcopenia are immobility, loss of independence, problems to perform activities of daily living and increased risk of falls and fractures (CRUZ-JENTOFT et al., 2010b). Furthermore during the aging process, the aging skeletal muscle shows an increase in ROS production. This is associated with accumulation of oxidized nucleic acids, proteins and fatty acids. In addition aging muscle cells have limited capacity for regeneration and this leads to oxidative stress and oxidative DNA damage (MCARDLE and JACKSON, 2000; JI, 2001). To counteract this multifactorial geriatric syndrome it is important to create adequate guidelines for healthy and active aging. Such guidelines should enable the elderly for physical activity, like endurance or strength training. It is also of great importance to investigate the benefits of physical activity in the elderly on a molecular basis.

How is the aging organism dealing with increased physical activity? What are the most interesting parameters to investigate and what are the changes?

These questions are of great interest in the current study.

2. OBJECTIVES

There are many reviews dealing with sarcopenia and what kinds of methods are suitable to counteract this progressive process of aging. Physical activity is an important issue in this context. Particularly strength training is often mentioned as an adequate method to counteract physical disability. The American College of Sport Medicine (ACSM) created guidelines for resistance training in the elderly. For improvement of muscle strength in the elderly they recommended eight to 10 strength-training exercises with 10-15 repetitions of each exercise twice to three times per week. This recommendation applies for healthy adult's ≥ 65 years and served as prevention guidelines against sarcopenia and frailty (HASKELL et al., 2007).

Beside the huge amount of papers about sarcopenia and the benefits of physical activity in the elderly, there are only a handful of trials dealing with the effect of strength training and DNA damage.

Therefore the Department of Nutritional Sciences and the Centre for Sport Sciences of the University of Vienna created a research platform to investigate the benefits of resistance training on muscle strength and the effect on DNA oxidation in older people. The current study represents a long term intervention which is planned for one and a half consecutive years with three periods of training.

The hypothesis of the study is: An adequate resistance training and nutrition reduces DNA damage in institutionalized elderly ≥65 years.

The main goal of this thesis is to compare the DNA oxidation status before and after three months of strength training.

3. LITERATURE SURVEY

3.1 SARCOPENIA

Numerous clinical trials have indicated that the development of sarcopenia is a multi-causal and very complex process. It is defined as an age related progressive decline in skeletal muscle strength and muscle mass (GOODPASTER et al., 2006; DELMONICO et al., 2007). This definition was first given by Irwin Rosenberg in 1988 (SCOTT et al., 2011). Nowadays there is an extended definition of sarcopenia which takes into account that the successive loss in skeletal muscle strength and muscle mass is also related to low physical activity in the elderly (JANSSEN et al., 2002; CRUZ-JENTOFT et al., 2010b). This diagnosis could be considered as a geriatric syndrome. Geriatric syndromes are only incompletely understood syndromes that result from interactions of age and various diseases, e.g. delirium, incontinence and falls (INOUYE et al., 2007; CRUZ-JENTOFT et al., 2010a). Sarcopenia has multiple contributive factors, such as malnutrition, aging, a sedentary lifestyle, drug treatments and several chronic diseases (PADDON-JONES et al., 2008; SAYER et al., 2008). Older people with the diagnosis of sarcopenia have to deal with mobility disorders, increased risk of falls and fractures, loss of independence and a disability to perform activities of daily living (LAURETANI et al., 2003; ROLLAND et al., 2008).

3.1.1 Development of Sarcopenia

Several factors seem to be involved in the development of sarcopenia. Lifestyle factors and molecular mechanisms and their interaction seem to be involved in the development of sarcopenia (BURTON and SUMUKADAS, 2010). Early lifestyle behaviors leading to a loss of muscle mass are smoking and physical inactivity. The same seems to be true later in life, with the addition of a protein-deficient diet and chronic diseases (ROLLAND et al., 2008; SCOTT et al., 2011). Therefore in older people a reduction in muscle mass is often observed. It may arise due to a combination of muscle fiber atrophy as well as of the loss of muscle fibers.

Increased muscle fatigability due to a denervation of motor units as well as a lower amount of satellite cells is also typical in older age. Satellite cells are involved in muscle regeneration and are therefore important for muscle function (**Fig.1**) (ERIM et al., 1999; AKIMA et al., 2001; THORNELL et al., 2003). Changes in the endocrine system are also observed including decreased levels of growth hormone (GH), insulin-like growth factor (IGF-1) and androgens. These hormones help to regulate growth and the development of skeletal muscle. Chronic inflammation also plays a role in the development of sarcopenia. Several studies have shown increased levels of pro inflammatory cytokines in the aging muscle (SCHAAP et al., 2006) .

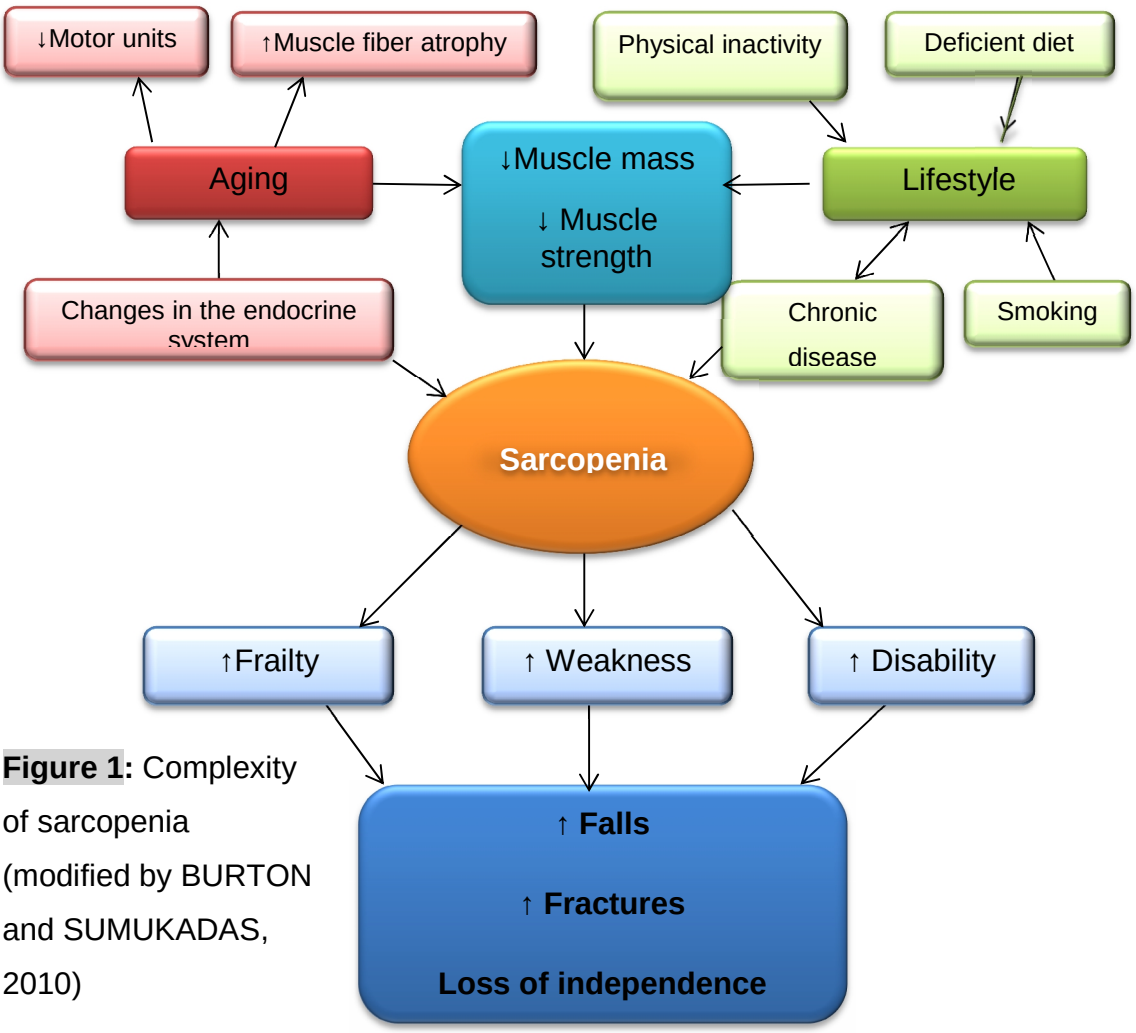

Figure 1: Complexity of sarcopenia (modified by BURTON and SUMUKADAS, 2010)

3.1.2 Methods for diagnosis of sarcopenia

The diagnosis of sarcopenia is made upon a few measurable parameters (**Tab.1**): muscle mass, muscle strength and physical activity (CRUZ-JENTOFT et al., 2010b).

3.1.2.1 Muscle mass

For the estimation of muscle mass or lean body mass body imaging techniques are approved methods. Mentionable are computed tomography (CT scan), magnetic resonance imaging (MRI), dual energy X-ray absorptiometry (DXA) and bioimpedance analysis (BIA). The golden standards for the evaluation of muscle mass in clinical trials are CT and MRI scans. These methods are very precise. An alternative method is DXA, which measures bone mineral density and fat as well as lean body mass. DXA is a whole body scan, which exposes the patient to minimal radiation. BIA is a less expensive method to distinguish the volume of fat and lean body tissue (CHIEN et al. 2008) .

3.1.2.2 Muscle strength

The progressive loss of muscle mass in the elderly leads to a loss of muscle strength. Both parameters influence physical activity in older persons (VISSER et al., 1998). Isometric handgrip strength is a simple and widely used technique for the determination of muscle strength. A reduction in handgrip strength is a clinical marker for reduced muscle mass and low physical activity. There is a linear correlation between handgrip strength and an inability to perform activities of daily living (LAURETANI et al., 2003; AL SNIH et al., 2004) .

3.1.2.3 Physical performance

Another diagnostic criteria for sarcopenia is physical performance. One common test is the Short Physical Performance Battery (SPPB). The SPPB is a standard method for the evaluation of balance, gait and strength. SPPB combines separate tests that have been used particularly in sarcopenia research. For instance a timed

4-meter walk and timed chair rise. Such tests predict the risk of disability in the future and can be useful for the identification of patients in the preclinical stage of sarcopenia (BURTON and SUMUKADAS, 2010).

Table 1: Diagnostic methods for sarcopenia (modified by BURTON and SUMUKADAS, 2010 and MORLEY, 2008)

Parameters	Methods	Comments
Muscle mass	Magnetic resonance imaging (MRI)	accurate, expensive
	Computed tomography (CT)	accurate, radiation exposure, expensive
	Dual energy X-ray absorptiometry (DXA)	Whole body scan, low radiation exposure
	Bioimpedance analysis (BIA)	Hydration status not exact, low reliability, less expensive
Muscle strength	Handgrip strength	Simple, good reproducibility
Physical performance	Short Physical Performance Battery (SPPB)	Validated method for older people

3.1.3 Prevention and treatment of sarcopenia

At present, management of sarcopenia is based on the combination of adequate physical activity and an adequate diet with a high amount of protein. It is well known that resistance exercises, as well as endurance training decrease the risk of development and progression of sarcopenia. Nevertheless resistance training

seems to be more effective to increase muscle strength and muscle mass (HUGHES et al., 2004; FRANKEL et al. 2006; ROUBENOFF, 2007). Recommendations for resistance training are: 8-10 exercises, with a resistance weight that allows 8-12 repetitions of each exercise. This resistance training should be performed on two days each week using the major muscle groups (HASKELL et al., 2007).

3.2 REACTIVE OXYGEN SPECIES AND DNA DAMAGE

The human organism is exposed to a variety of reactive oxygen species (ROS). ROS either arise through cellular metabolism, such as aerobic metabolism or they are reduced by exogenous sources. These genotoxic agents are often responsible for the reduction of the antioxidant capacity and the induction of oxidative DNA damage, such as single strand breaks and oxidized bases. The emerging DNA breaks lead to genetic instability and various mutations (DIZDAROGLU, 2012; MAGNANDER and ELMROTH, 2012). DNA damage caused by ROS increases with advanced aging (RADAK et al., 2011).

3.2.1 Reactive oxygen species (ROS)

Free radicals, such as ROS are highly reactive molecules (**Tab.2**). ROS arise from the addition of a single electron to the oxygen molecule. It is well known that high ROS concentrations are associated with irreversible transformation or complete damage in nucleic acids, lipids and proteins. ROS are linked to cancer, cardiovascular disease, atherosclerosis and aging. But they also play an essential role in physiological and biosynthetic processes, such as redox regulation of protein phosphorylation and thyroid hormone production (ALFADDA and SALLAM, 2012; BRIEGER et al., 2012).

Table 2: Reactive Oxygen Species [modified by Preiser, 2012]

Free Radicals		**Nonradicals**	
Superoxide anion	$O_2^{\cdot}$	Hydrogen peroxide	H_2O_2
Hydroxyl radical	$^{\cdot}OH$	Nitric oxide radical	$NO^{\cdot}$
Peroxynitrite	$ONOO^-$	Hypochloric acid	HOCl

Most cells and tissues convert molecular oxygen into superoxide anion ($O_2^{\cdot-}$) in the mitochondrial respiratory chain (FULLE et al., 2004). The typical initial reaction in the development of ROS is the conversion from oxygen (O_2) to superoxide ($O_2^{\cdot-}$) by NADPH-oxidase. During further reactions superoxide anion is catalysed by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). In comparison to superoxide anion, hydrogen peroxide (H_2O_2) is longer lasting and membrane permeable. Hydrogen peroxide (H_2O_2) can be transformed enzymatically by glutathione peroxidase (GPx) or catalase into H_2O or by peroxidase into hypochlorous acid (HOCl). It is also nonenzymatically converted in the presence of iron into hydroxyl radical ($^{\cdot}OH^-$) (**Fig.2**). The reaction between superoxide anion ($O_2^{\cdot}$) and nitric oxide ($NO^{\cdot}$) lead to the generation of highly reactive peroxynitrite ($ONOO^-$) (BRIEGER et al., 2012; PREISER, 2012).

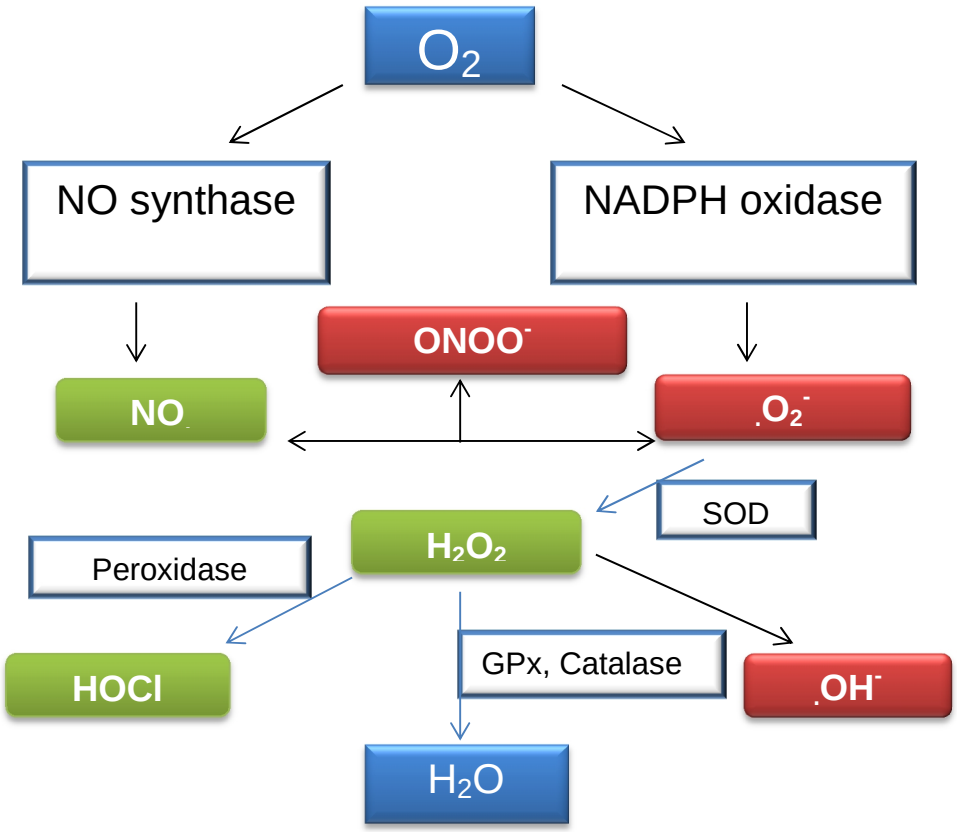

Figure 2: Mechanism of ROS generation, (modified by PREISER, 2012)

3.2.2 Essential role of reactive oxygen species in the aging muscle

Aging constitutes an extensive biological process. It is characterized by a decline in physiological and biochemical functions. This process entails several changes which include a loss of muscle mass characterized by a reduction in muscle fiber diameters, increased production of ROS, decreased antioxidant capacity and a reduced capacity to use oxygen (BUONOCORE et al., 2011; DORIA et al., 2012).

In muscle fibers ROS are produced in different cellular sites: mitochondria, sarcoplasmic reticulum, sarcolemma and plasma membrane. They are usually moved to the cytosol muscle cells (POWERS and JACKSON, 2008). ROS produced by mitochondria are able to induce oxidative damage to mitochondrial proteins, membranes and mtDNA (mitochondrial DNA).The consequence of this

oxidative process is the impaired ability of mitochondria to carry out their metabolic functions, such as fatty acid oxidation and amino acid metabolism. This results in the synthesis of oxidized lipids, damaged proteins and mtDNA mutations which cause mitochondrial and cellular failure (HARMAN, 2006).

Further consequences are the activation of the apoptotic machinery by cytochrome *c*. It is well known that apoptosis of different cells and tissues is increased during the aging process. This fact contributes considerably to cell loss and the pathogenesis of several age-related diseases, such as loss of lean body mass (FANÒ et al., 2001; MARZETTI et al., 2008). Aging is associated with decreased utilization of enzymatic and nonenzymatic antioxidant systems that convert ROS into inactive species. This redox imbalance constitutes the main trigger for the imbalance between protein synthesis and degeneration that leads to muscle atrophy (MOYLAN and REID, 2007; POWERS et al., 2010). Skeletal muscle constitutes the major consumer of oxygen in our body (MCARDLE et al., 2001). Therefore skeletal muscle constitutes the tissue with the highest production of ROS in comparison to other tissues (FULLE et al., 2004).

ROS are not only involved in muscle damage, but also affect the contractile function of skeletal muscle (PELLEGRINO et al., 2011).

During contractile activity there is an increased generation of superoxide and nitric oxide (BUONOCORE et al., 2011). Taken together all mentioned processes play a tremendous role in the mitochondrial theory of aging (CHABI et al., 2008).

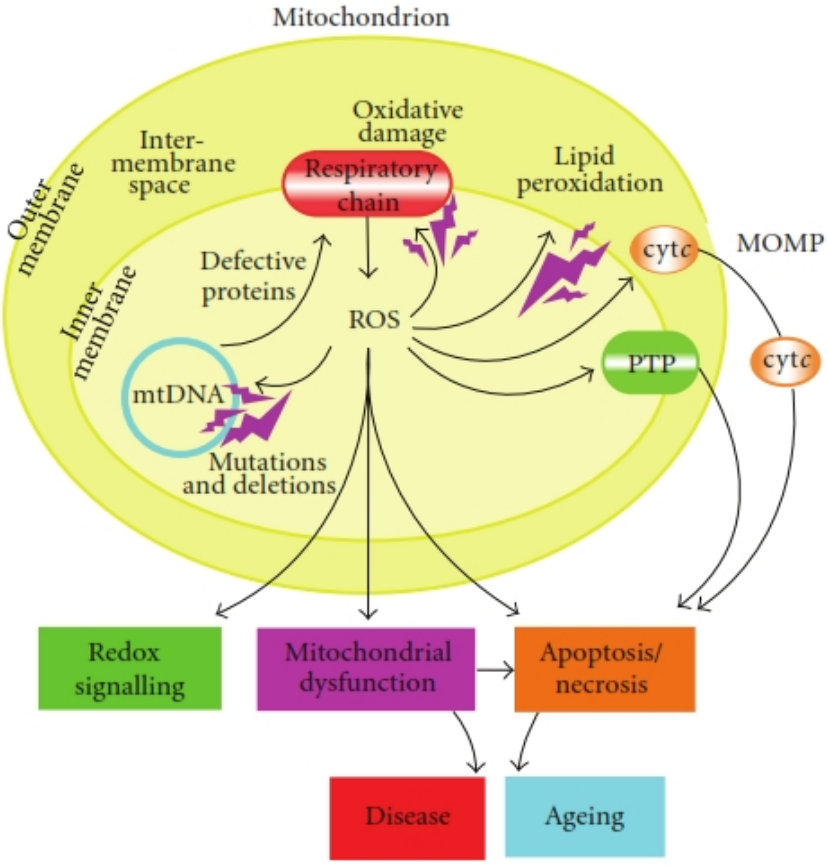

Figure 3: Mitochondrial ROS production (MURPHY, 2009)

3.3 PHYSICAL ACTIVITY, AEROBIC EXERCISE AND STRENGTH TRAINING IN THE ELDERLY

3.3.1 Physical activity in the elderly

The term physical activity (PA) is defined as any physical movement which is caused by skeletal muscle contraction and which considerably increases energy expenditure above basal level. Physical movement can be classified into two categories: baseline activity and health-enhancing physical activity. Baseline physical activities refer to performances of daily life, including standing, walking slowly and lifting low weight objects. Individuals doing only this kind of activity are

considered to be inactive. The second category, health-enhancing physical activity sums up activities such as brisk walking, jumping rope, dancing, lifting weights, doing yoga and several more. The combination of baseline activity and health-enhancing physical activity produces substantial health benefits. There is a further classification of physical activity: mode and intensity. Mode is the type of activity which is performed, such as weight lifting, Nordic walking, biking and running. The classification intensity is described as relative and absolute intensity. It indicates the effort which PA is performed (U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES, 2013).

3.3.1.1 Physical activity and exercise

Physical activity and exercise differ in their definitions. The term exercise is defined as the bodily performance doing during leisure time and is a planned, structured and repetitive PA. The purpose of this bodily activity is to improve the physical fitness or health (FREIBERGER et al., 2011). Therefore elderly people may perform daily activities such as walking or gardening but they do not perform any exercise (BRACH et al., 2004; BUCHNER, 2009). Physical exercise is defined by mode, intensity and frequency (BLAIR, 2007).

Further variables are important to modify muscle wasting and decline: physical fitness and physical function (KEYSOR, 2003). Physical fitness is characterized by several physiological components including: endurance, skeletal muscle strength, flexibility and body composition (U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES, 2013). Physical function is considered as the individual‘s ability to perform daily activities. It is characterized by physical, cognitive and mental components (BRACH et al., 2004).

3.3.2 Aerobic exercise in the elderly

The maximal aerobic exercise capacity is defined as the maximal oxygen consumption during aerobic exercise (VO_2max). When aerobic exercise is regularly performed VO_2max is increased through following mechanisms: increased stroke

volume, increased and improved capacity to use oxygen by skeletal muscle. During aging VO_2max is decreased approximately 1%/year between the ages of 20 and 70 years. Responsible for this decline is physical inactivity, decreased cardiac function and reduced muscle mass (WILLIAM, 2000). Aerobic exercise can affect the aging muscle in a positive way (JOHNSTON et al., 2008). One positive effect is an increase in number of skeletal muscle mitochondria (LANZA et al., 2008). This seems to be a result of exercise-induced increase in the transcription of muscle mitochondrial genes (HAWLEY et al., 2006). The transcriptional co-activator peroxisome proliferator-activated receptor gamma co-activator 1-α (PGC-1α) is considered to play an essential role in the regulation of mitochondrial biogenesis (LIRA et al., 2010). The expression and activation of PGC-1α in skeletal muscle rises during acute exercise (LITTLE et al., 2010). An increase in muscle mitochondrial content and improved mitochondrial function has beneficial effects on metabolism, endurance capacity and oxidative stress (SAFDAR et al., 2010). Furthermore aerobic training improves skeletal muscle insulin sensitivity (HAWLEY, 2004). Insulin resistance is often seen during aging and may cause skeletal muscle wasting by reducing insulin-like growth factors (HILDRUM et al., 2007; GREENHAFF et al., 2008).

3.3.3 Strength training- beneficial effects in the elderly

A continuous decline in muscle strength and muscle mass is observed during the 3th decade until about the 5th decade of life. In the 6th decade of life a progressive non-linear reduction by 15 % is observed. At the 8th decade of life reduction in muscle mass and strength increases up to 30% (FAULKNER et al., 2007).

The age-related changes are: reduction in motor capacity, decrease in muscle type I and type II fibers especially in the lower extremity and impairments to mechanical muscle function (Aagaard et al., 2010).The results of such changes are a functional deficit in strength and balance, as well as in gait safety. Furthermore the risk of falls, injuries and degenerative diseases is increased (FAULKNER et al., 2007).

The incidence of falls and fractures rises in the 5th decade of life. In the 6th decade of life 30% of people fall at least once a year (ORR et al., 2008) . There are several studies that demonstrate that strength training can counteract age related impairments by an increase in muscle mass (PETRELLA and CHUDYK, 2008; MAYER et al., 2011). Strength exercise has positive effects on muscle hypertrophy and on muscle strength in older people (CANDOW et al., 2006; CANDOW et al., 2008). An increase in muscle mass resulting from resistance exercise, leads to an increase in muscle protein synthesis, satellite cell activation and proliferation, anabolic hormone production and a decrease in catabolic cytokine activity (SMILIOS et al., 2007; VERDIJK et al., 2009). Furthermore continuous strength training increases the cross-sectional area of type I and type II muscle fibers. This leads to a clinically significant increase in muscle strength (BROSE et al., 2003; TARNOPOLSKY et al., 2007).

In a current comparative study, Candow et al. demonstrated that short-term (22 weeks; 3 days per week) of heavy resistance training in healthy older men (60-71 years, N=17) is sufficient to overcome age-related deficits in muscle mass and strength (CANDOW et al., 2011). Further laboratory-based trials confirm this investigation and in addition indicate that 20 to 30 minutes of resistance training, 2 to 3 times per week, has positive effects on sarcopenia, diabetes, osteoporosis and cardiovascular disorders (MARTYN-ST JAMES and CARROLL, 2006; DANIELS et al., 2008; LIU and LATHAM, 2009). This data shows that resistance training is an effective intervention for gaining muscle mass and muscle strength. This aspect is also responsible for the improvement of quality of life of older people (PETERSON et al., 2011; FORBES et al., 2012). Activity improves the ability to walk long distances, leads to better performance and a higher mobility (LIU and LATHAM, 2009).

3.3.4 Recommendation for physical exercise in the elderly

In 1995 the Centres for Disease Control and Prevention (CDC) and the American College of Sports Medicine (ACSM) published a recommendation of moderate-intensity physical activity for all healthy adults aged 18 to 65 years. The aim of this recommendation was to provide a public message for an active and healthy living (PATE et al., 1995). Since 2007 exists an updated recommendation for physical activity from the American College of Sports Medicine (ACSM) and the American Heart Association (AHA). The updated recommendation is fundamentally unchanged from the 1995 recommendation but there are few improvements: Clarification in the advised frequency for moderate-intensity of physical activities; Incorporation of high-intensity training; Moderate- and high-intensity training are both beneficial for health and muscle strength. Finally the new recommendation points out that physical activity above the advised amount provides even greater health benefits (HASKELL et al., 2007).

3.3.4.1 Recommendation for aerobic exercise

For healthy adults aged 18-65 years it is recommended to practise moderate-intensity aerobic physical activity (e.g. walking briskly) for a minimum of 30 min on five days each week or vigorous-intensity aerobic (e.g. jogging) activity for a minimum of 20 min on three days each week. The minimum levels of this recommendation are for untrained or frail individuals (HASKELL et al., 2007).

3.3.4.2 Recommendation for strength training

Elderly people who do not perform additional training to their activities of daily life will lose body strength to a disproportionate degree (MAYER et al., 2011). Therefore it is recommended to perform 8-10 strength exercises for the major muscle groups on two to three times a week (**Tab.3**). For muscle hypertrophy, it is advised to use weights that allow 8-12 repetitions of each exercise resulting in substantial muscle fatigue.

Muscle strength exercises include a progressive weight training program that uses the major muscle groups. Typical devices for strength training are free weights, resistance bands and participant´s own body weight (HASKELL et al., 2007; MAYER et al., 2011). Continuous resistance training can improve muscular strength and endurance by 25% to 100% (POLLOCK et al., 2000).

Table 3: Different kinds of strength training, (modified by MAYER et al., 2011)

Purposes	Training and Performance	Effects of Training
Increase in muscle strength	8–12 repetitions per muscle group in 70–85 % of the one-repetition max., 3 sets; 2–3 training units per week	Increase in muscle mass
	Up to 8 repetitions per muscle group with intensities of more than 80% of the one repetition max.; 3-5 sets; 3 training units per week	Training of intramuscular coordination
Prevention of falls and injuries	Several repetitions; up to daily training units; high speed of movement	Optimizing postural control; training of intermuscular coordination
Reduction of sarcopenia	8–12 repetitions per muscle group in 60–80% of the one-repetition max.; 3 sets, 3 training units per week	Increase in muscle mass

3.4 TRAINING AND OXIDATIVE DNA DAMAGE IN THE ELDERLY

Excessive physical training on its own can lead to ROS production and therefore facilitates oxidative DNA damage. Yet moderate and frequent training boosts antioxidative pathways. Therefore the body is able to repel against ROS stress more effectively (RADÁK et al., 2002).

Aging in skeletal muscles is associated with increased ROS production. This leads to accumulation of oxidized proteins, fatty acids and nucleic acids. Secondly this process is attended by a higher rate of damage and inflammation to muscle tissue. Particularly inflammation is associated with oxidative stress. Aging muscle cells have a limited capacity for regeneration, due to the aging process. Because of this fact it seems to be obvious that physical exercises should lead to more oxidative damage in the elderly than in younger individuals. Paradoxically the positive effect of physical training is also given in later phases in one's life. The body is profiting by adaptions in the cellular antioxidant defense systems induced by physical training. Moreover training helps to improve muscle strength and endurance, which on its own leads to a reduction in inflammatory processed and helps to prevent injuries (MCARDLE and JACKSON, 2000; Ji, 2001).

Frequent physical activity also leads to a reduction in the prevalence of a variety of diseases, such as cancer, rheumatic diseases, diabetes and sarcopenia. Also on the physical level it helps to improve the cardiovascular system or leads to better cognitive processing and as mentioned above increases muscular mass and strength (RADÁK et al., 2002).

Radák et al. were able to show in their study that frequent physical training reduces age-associated increase in 8-Oxo-2'-deoxyguanosine (8-OHdG) in skeletal muscle cells (RADÁK et al., 2002). 8-OHdG is an oxidized derivate of deoxyguanosine that is thought to be the main product of DNA oxidation. Further it is a parameter that can be easily measured and moreover is a good biomarker for oxidative stress (BARZILAI and YAMAMOTO, 2004).

Furthermore they showed that exercise training led to an enhanced resistance against oxidative stress in the aging muscle. Oxidative DNA damage, as well as the susceptibility for oxidative damage of proteins was diminished (RADÁK et al., 2002).

Parise et al. conducted a trial with very similar results. 28 women and men at a mean age of 68,5 years performed a regular whole body strength training for 14 weeks. After the intervention the concentration of 8-OHdG sites in DNA was significantly reduced (Fig.4) (PARISE et al., 2005).

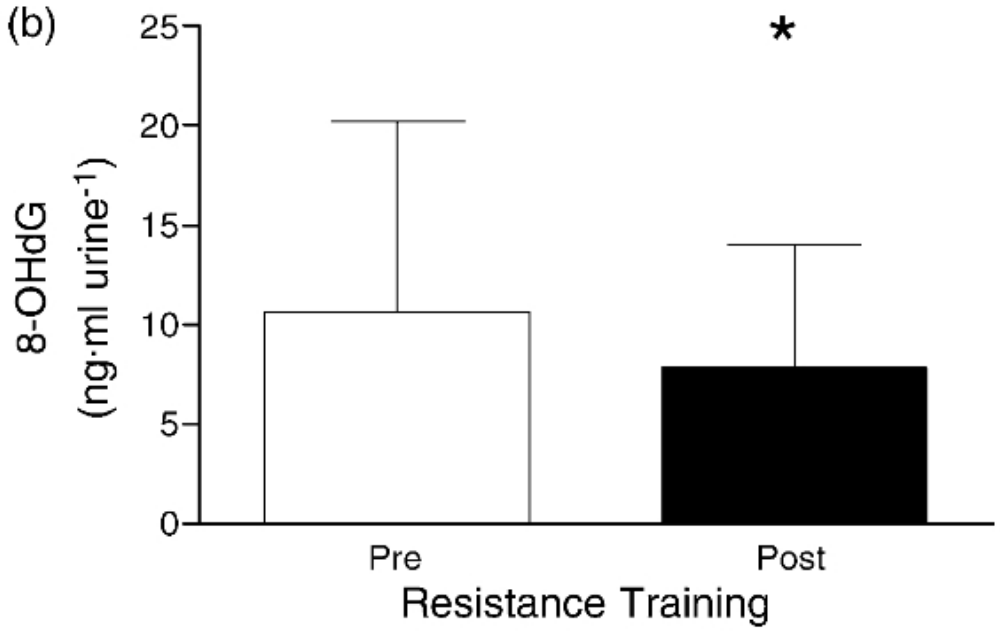

Figure 4: Reduction of 8-OHdG levels after 14 weeks resistance training in the elderly (PARISE et al., 2005)

3.5 SINGLE CELL GEL ELECTROPHORESIS (SCGE)

The Single Cell Gel Electrophoresis (SCGE or comet assay) was first used in the 1970s (COOK et al., 1976). Nowadays it is a widely accepted method for measuring DNA damage in mammalian cells. SCGE is used in a variety of applications, e.g. assay for DNA damage, human biomonitoring and ecological monitoring (COLLINS, 2004). The term “comet assay” results from the similarity of the image obtained from the microscope to the astronomical body (**Fig.4**). The comet-head consists of intact DNA and the comet-tail contains damaged or broken pieces of DNA (LIAO et al., 2009). Such a “comet” shape is induced during the electrophoresis process, when the DNA migrates out of the cell (FAIRBAIRN et al., 1995).

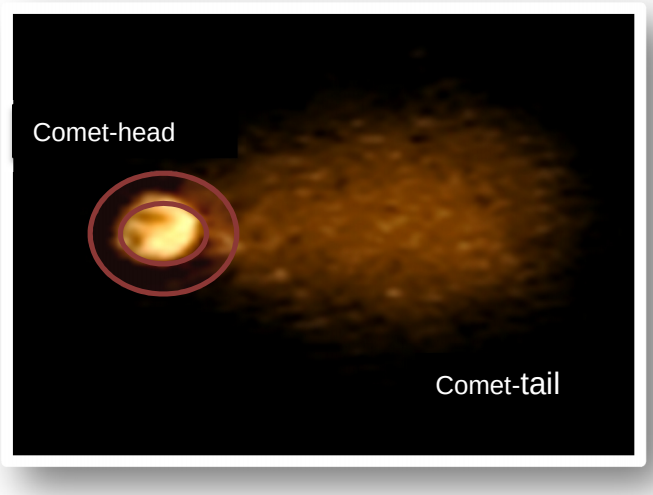

Figure 5: Image resulting from the fluorescence microscope (own results)

3.5.1 The development of SCGE- historical aspects

Peter Cook et al. were the first who were engaged in the development of a method to investigate a nuclear structure based on the lysis of cells with nonionic detergent and high-molarity sodium chloride. The treatment with detergents causes removal of cell membranes, cytoplasm and nucleoplasm (COLLINS, 2004).

In addition, the high salt concentration leads to a disruption of nucleosomes and to a solution of histones. After this process you get a nucleoid which consists of a nuclear matrix, proteins and a negative supercoiled DNA (COLLINS, 2004).

The first demonstration of DNA tails with a microscope photometer was in 1984. Ostling and Johanson created a method in order to investigate DNA damage at a single cell level. The procedure was similar to the method of Cook et al. The difference was that Ostling and Johanson tried to detect the tail DNA by using a fluorescent dye (acridine orange) for staining the liberated DNA. This procedure was performed under neutral conditions (OSTLING and JOHANSON, 1984).

Four years after developing the method by Ostling and Johanson, Singh et al. modified the procedure involving cell treatment at high pH level (pH>13).This induces unwinding of DNA under alkaline conditions. The investigation group around Singh lysed cells at pH 10 with 2.5 M NaCl, Triton X-100 and Sarkosyl (nowadays frequently omitted from the lysis solution) for 1h. Then treatment with alkali (0.3M NaOH) followed and finally electrophoresis at pH >13 (SINGH et al., 1988).

Only two years later Olive et al. demonstrated another version of alkaline SCGE. They used a considerably weak alkali solution (0.03M NaOH) for cell lysis, also 1 h before electrophoresis (OLIVE et al., 1990).

In summary, alkaline treatment leads to denaturation of DNA which results in single-strand or double-strand breaks. There is a corresponding neutral method, which doesn’t induce DNA denaturation. The rate of elution depends here on the frequency of double-strand breaks (COLLINS et al., 2008).

3.5.1.1 Updated principle of SCGE

Today, the SCGE assay consists of following steps (**Tab.4**): embedding cells in agarose; lysis treatment (with detergent and high salt); treatment with H_2O_2 (for ROS generation); treatment with lesion-specific enzymes ENDOIII (endonuclease III) and FPG (formamidopyrimidine-DNA glycosylase) for endogenous formation of oxidized pyrimidines and purines; alkaline treatment; electrophoresis; neutralization and washing (with H_2O bidest) and finally detection of DNA tails by fluorescence microscopy after staining with a DNA-binding dye (Ethidium bromide) (COLLINS, 2004, 2009).

Table 4: Different treatments during the SCGE procedure, (modified by HOELZL et al., 2005)

Procedure	Endpoint
Lysis of cell and nucleoid membrane	Backwardness of DNA
Treatment of cells with ROS-generating chemicals, such as H_2O_2	Altered sensitivity to exogenous ROS
Treatment of nuclei with lesion-specific enzymes FPG and ENDO III	Endogenous formation of oxidized pyrimidines and purines
DNA unwinding under alkaline conditions	Formation of single-strand breaks

The treatment with enzymes, such as FPG and ENDOIII is a neat way to detect oxidized pyrimidines and to identify the major purine oxidation product 8-oxoguanine (COLLINS, 2004; LIAO et al., 2009). The exposure of intact cells to ROS-generating chemicals such as H_2O_2 indicates the changes of the sensitivity of cells (e.g. lymphocytes) towards exogenous oxidative DNA damage. This treatment gives information about the antioxidant status (HOELZL et al., 2009).

3.5.1.2 The advantages of SCGE

The SCGE is a simple and low-priced technique. This method can be applied to diverse tissues of interest (e.g. lymphocytes separated from whole blood). Further advantages are: detection of multiple classes of DNA damage and production of data at single cell level. The results can be obtained in a few hours, which pose another benefit. Taken together all this aspects make SCGE a suitable technique that can be used in different clinical trials (HARTMANN et al., 2003; MCKENNA et al., 2008), including cancer studies, lifestyle and dietary studies (FIKROVÁ et al., 2011).

3.5.2 Application of SCGE in human intervention trials

In the last twenty years SCGE was used in numerous intervention trials, especially in dietary and lifestyle studies. The first application of SCGE in a human intervention trial with vitamins was in 1996.

This study was conducted by Duthie et al; the aim was to investigate the influence of dietary factors on DNA stability (DUTHIE et al., 1996; HOELZL et al., 2009). The most recent intervention trials use the SCGE protocols developed by Collins et al. (as described in *3.4.1.1*) (COLLINS, 2004; HOELZL et al., 2009).The majority of investigations are carried out with healthy individuals. Some recent studies were conducted with subjects under oxidative stress, e.g. healthy persons after exercise or patients with diabetes after consumption of vegetables (HOELZL et al., 2005).

3.5.2.1 Dietary intervention

The SCGE is a very useful method for investigating effects of several nutrients or micronutrients at the level of DNA damage in humans (Jenkinson et al., 1999).There are several studies which investigate the potential protective effects of antioxidants on DNA damage (FIKROVÁ et al., 2011).

The beneficial effects of *in vivo* supplementation of antioxidants or of diets rich in antioxidants are usually demonstrated in lymphocytes. Studies show a decrease in the endogenous formation of oxidized purines and pyrimidines and a decreased sensitivity to H_2O_2 induced DNA damage in vitro (DUTHIE et al., 1996; HOELZL et al., 2005). Previous human dietary intervention trials focused especially on the protective effects of isolated vitamins (A, C, E) and carotenoids. Recent studies try to investigate the antioxidative effects of fruit juices, coffee and a diet rich in vegetables.

In an intervention trial Misik et al. demonstrated the positive effects of coffee consumption on oxidative DNA damage. They conducted their cross-over study with 38 young and healthy individuals. The participants consumed 800 ml coffee or water over 5 consecutive days. SCGE assay was used for the measurement of oxidized DNA damage in peripheral human lymphocytes. The research group reveals that after the intake of paper filtered coffee the extent of DNA migration due to formation of oxidized purines was reduced by 12.3%. This observation leads to the assumption that ingredients of coffee lead to a prevention of endogenous formation of oxidative DNA damage in human individuals (MIŠÍK et al., 2010).

Another intervention trial by Müllner et al. tried to investigate the effect of a healthy diet on DNA oxidation in diabetics and non-diabetics. This intervention study was executed with 76 diabetics and 21 non-diabetics. The intervention group received information about a healthy diet and additionally 300g of vegetables and 25 ml PUFA- rich plant oil per day. The control group received only information about healthy diet. The results show that diet rich in vegetables and PUFA-rich oil lead to a significant increase in plasma antioxidant concentrations. Besides there was a significant reduction in DNA strand breaks in diabetic persons in the intervention group. After 4 weeks there was a reduction of 13.8 % of DNA strand breaks and after 8 weeks a reduction of 17.1%. The reduction of DNA strand breaks was significant in diabetics, but not in non-diabetics. In the control group no significant effects were observed (MÜLLNER et al., 2013) .

3.5.2.2 Exercise intervention

It is well known that high concentrations of ROS lead to oxidative modifications of macromolecules and to tissue damage. Moderate concentrations of ROS play in turn an essential role in normal cell signaling (TRAUSTADÓTTIR et al., 2012). On the one hand some evidence suggests that high intensity physical activity (e.g. ultra marathon) is associated with increased DNA damage and on the other hand moderate physical activity is associated with an increase in DNA repair (CASH et al., 2013).

In the last decade few studies were performed trying to investigate the influence of exercise training on DNA damage or repair mechanisms. For measurement of DNA damage several trials used the SCGE.

Hartmann et al. conducted one of the first studies with three participants who passed a very fast run to exhaustion on a treadmill and a second run on individual constant speed. The research group showed that exhaustive training induced an increase in DNA damage. DNA damage was measured in peripheral blood cells with the SCGE (HARTMANN et al., 1994). The study population was very small therefore you cannot take a very precise conclusion.

Currently Cash et al. demonstrated in a study with 220 healthy elderly (50-76 years) that physical activity was unrelated to the formation of DNA damage but associated with increased DNA repair. They measured the DNA status after different activity intensities: total activity, moderate-plus high intensity activity and high intensity activity. Once more SCGE assay was used (CASH et al., 2013).

3.5.2.3 Summary of human intervention trials

Since the first application of SCGE in a human intervention trail in 1996, more than 84 intervention trials have used the SCGE technique to this day.

In order to receive information about the antioxidant status, several studies use the treatment of cells with H_2O_2 or with gamma radiation. Studies using only lysis treatment (standard conditions) are only able to detect a decrease in DNA migration of 30%. Cells treated with H_2O_2, FPG and ENDOIII demonstrate a significant protection towards ROS and a significant decrease in formation of oxidized bases (HOELZL et al., 2009).

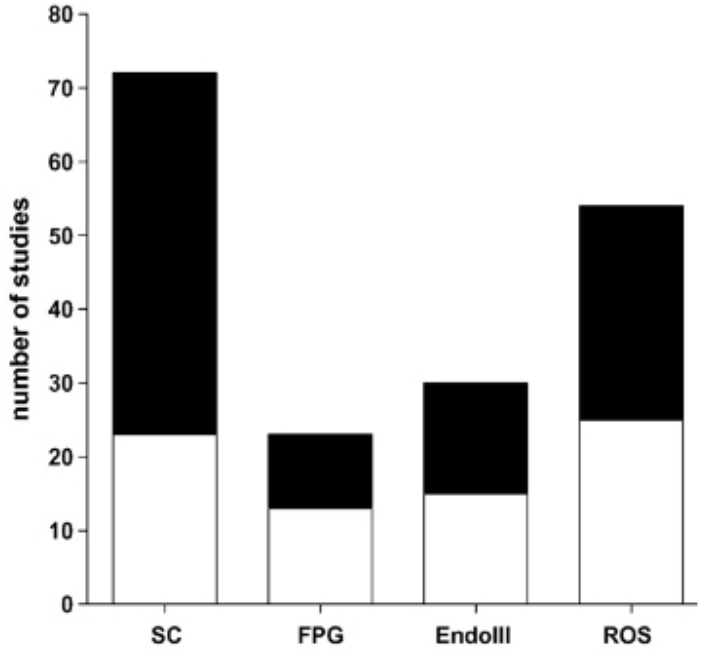

Figure 6: Evaluation of results received in SCGE trails. SC: standard conditions, FPG and EndoIII: enzymes, ROS: damage induced by H2O2 or radiation; grey bars: negative results, white bars: positive results (HOELZL et al., 2009).

Heterogeneity in the results of many studies could be due to a small number of participants, short duration of intervention and the use of different SCGE protocols.

SCGE is frequently used in dietary intervention studies, but only scarcely in exercise intervention studies. There are not many data about the antioxidative effect of regular, moderate physical exercise over a longer time.

4. MATERIALS AND METHODS

4.1 STUDY DESIGN

The present study is part of the research platform “Active Ageing”. It is collaboration between the Department of Nutritional Sciences and the Centre for Sport Sciences of the University of Vienna.

The randomised intervention trial comprised in the first and second period altogether 31 elderly above the age of 65 years of age. The subjects are unblinded, but the outcome assessors are masked.

The study was reviewed and finally approved by the ethics committee of the City of Vienna. This research project is scheduled for one and a half years. There are four periods of biochemical analysis. Biochemical pre-tests started in October 2011. The method applied was single cell gel electrophoresis (SCGE).

After the pre-tests the elderly started their strength training two days a week. In the first six months they passed their exercise under the supervision of sport experts. In the following six months they started to practise their exercise once a week on their own and the other day they completed their training under supervision. At the end of month 12 the elderly practised the strength training for the next six months completely on their own. The three study groups were: Group 1: physical exercise, Group 2: physical exercise and dietary supplementation, Group 3: cognitive training (**Fig. 7**).

Dietary supplementation in the second group was accomplished by additionally offering a supplement with high quality protein content (FortiFit). The third group only participated in a cognitive training and was therefore considered as a control group for DNA damage.

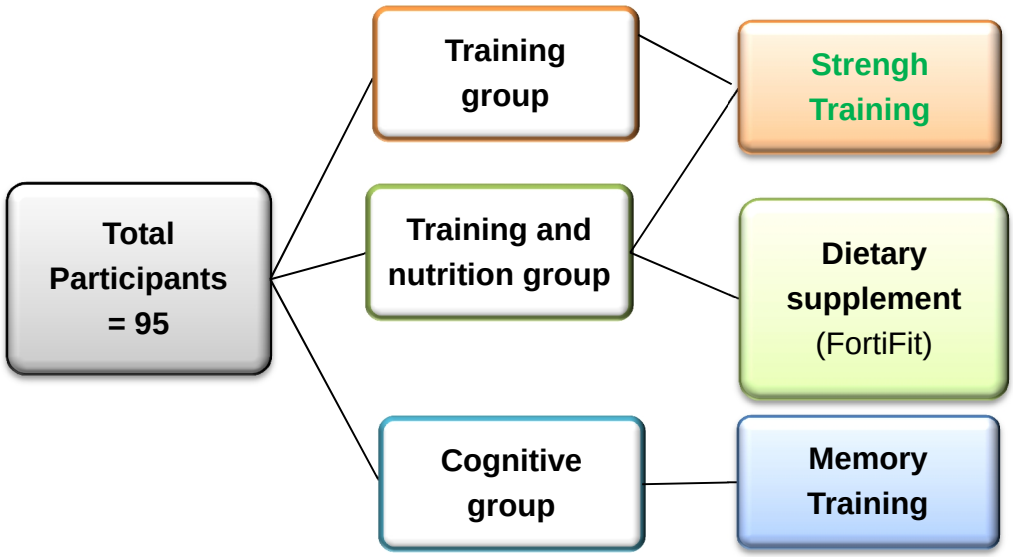

Figure 7: Study groups and intervention method

4.2 SUBJECTS

The participants were recruited in five homes of the Kuratorium Wiener Pensionistenheim (KWA). The participation was exclusively by choice. Interested subjects that met the inclusion criteria (**Tab.5**) had to pass medical tests (**Tab.6**) before they were able to enter the study.

All subjects gave their written consent for the participation. The assignment to the groups was randomised and unknown for the assessor.

4.2.1 Inclusion criteria, exclusion criteria and medical test

Table 5: Inclusion criteria

Inclusion criteria
o Men and women > 65 years of age o Mini–mental state examination >23 points o Ability to walk a distance of 10 meters on their own o Short Physical Performance Battery (SPPB) > 4points

Table 6: Exclusion criteria

Exclusion criteria
o Chronic disease o Severe cardiovascular disease o Diabetic retinopathy o Regular strength training in the last six months o Apparent osteoporosis o Anticoagulant medication o Ongoing corticosteroid therapy o No written comprehension

Table 7: Medical test

Medical tests
o Assessment of anthropometrical data (BMI, body fat, body weight, body height) o Functional strength tests o Muscle biopsy (voluntarily) o Magnetic resonance imaging o Ultrasound o Blood collection

4.3 INTERVENTION AND CONTROL GROUP

4.3.1 Physical activity intervention

The training program for exercise intervention was created by following the guidelines for adults above the age of 65. These guidelines are developed by the *American College of Sports Medicine* (ACSM) (HASKELL et al., 2007). The physical activity group completed every week two sessions of 6-10 progressive strength-training exercises, with 8-10 (60-80% one repetition maximum= 1RM) repetitions per exercise. The strength training was only performed with the help of an elastic band, a chair and the own body weight.

The exercise program was split in three parts:

1. 10 minutes warm-up with proprioception and balance exercises
2. 30 minutes strength-training exercises
3. 10 minute cool down

Altogether one training session took 50 minutes. Intensity was increased as soon as participants completed their exercise with 12 repetitions without symptoms of fatigue.

4.3.2 Training and Nutrition intervention:

One study group received besides physical training also a dietary intervention. Participants under nutritional intervention applied a certain dietary supplement (Nutricia) with a high amount of leucine and other essential amino acids, as well as antioxidative vitamins. It is supposed to facilitate muscle protein biosynthesis and to support muscle function and strength (NUTRICIA, 2012).

Table 8: FortiFit Ingredients (NUTRICIA, 2012)

Ingredients	FortiFit /100 ml	FortiFit /150 ml 1 portion
Energy (kcal)	100	150
Protein (g)	13,8	20,7
Whey Protein (g)	13,1	19,7
Leucine (g)	2	3
Essential amino acids (g)	>6,6	>10
Carbohydrates (g)	6,2	9,3
Fat (g)	2,0	3,0
Fibres (g)	0,8	1,2

Dietary supplements were served to the subjects once a day as a shake in addition to their regular diet. During the physical activity days subjects received a further shake after their workout.

4.3.3 Control group- Cognitive group:

The cognitive group started at the same time with their memory training. This should avoid bias in the study design due to positive effects of social connection in the intervention groups.

4.4 METHODOLOGY

The assay used in this trial is the single cell gel electrophoresis (SCGE) (**Fig.8**).This is a sensitive and simple method for the detection of single- and double- strand breaks in cellular DNA (SINGH et al., 1988). Typically cells used for SCGE are human lymphocytes. It is based on the loosening of DNA supercoiling by strand breaks and subsequent extension of DNA to form a 'comet tail' under alkaline electrophoresis (COLLINS, 2004; COLLINS et al., 2008). For this purpose human lymphocytes are embedded in agarose on a microscope slide. Then cells are lysed in a suitable solution including 2.5m NaCl. Lysis leads to dissolution of membranes, proteins and histones. The DNA leaves the cell supercoiled, attached to the nuclear matrix but without histones. Incubation in an alkaline solution induces DNA loops. The damaged DNA moves in the direction of the anode. This is detectable under a fluorescence microscope with ethidium bromide as a staining reagent (**Fig.5**).

In the last decade SCGE has become one of the standard methods for measuring DNA damage. This versatile method is used in human biomonitoring trials with environmental or job-related exposure to various genotoxic agents, oxidative stress, radiation or chemical substance (DUSINSKA and COLLINS, 2008)**.**

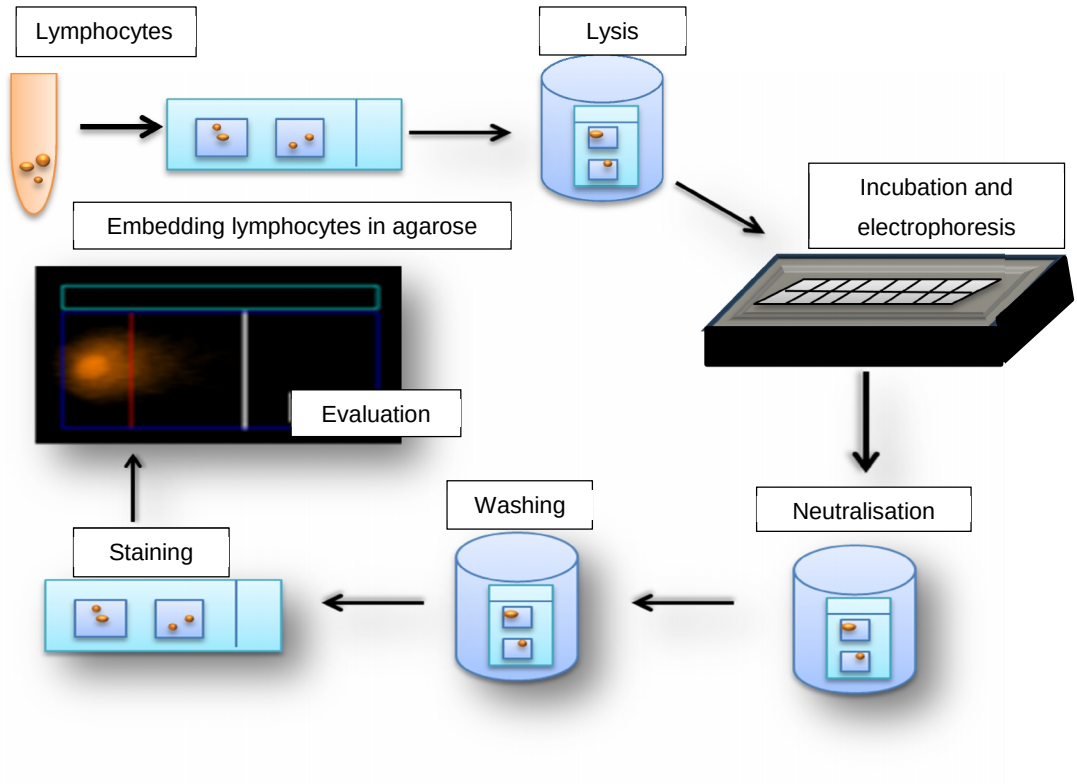

Figure 8: Principle of SCGE

4.4.1 Chemicals and working equipment used for SCGE

Table 9: Equipment used for the SCGE

Equipment	Manufacturer	Product no.
pH meter	Metrohm GmbH	827ph lab
Calibration solution	Metrohm GmbH	62307.100/110
Countess-automated cell counter	Invitrogen life technologies	C10227
Countess cell counting chamber slides	Invitrogen life technologies	C10283

Electrophoresis	Biozym Scientific GmbH	CS 08091040/ CS 0809103
Power supply	PEQLAB Biotechnologies GMBH	S/N85239
SCGE microscope	Zeiss Germany	038-03241
SCGE lamp	Prior Scientific (-Lumen 200)	L200/D
SCGE program	Komet 5.5 image analysis system (Kineting Imaging)	5.5144
Microscope slides	VWR	631-1551

Table 10: Chemicals and Reagents used for SCGE

Reagents	Abbreviation	Manufacturer	Product no.
Dulbecco´s Phosphate Buffered Saline	PBS	Sigma Life Science	D8537
Ultrapure NMA Agarose	NMA	Invitrogen life technologies	16500-100
Ultrapure LMP Agarose	LMA	Invitrogen life technologies	16520-050
Sodium Chloride	NaCl	Sigma-Aldrich	71380
Ethylendiamine-tetraacetic acid	EDTA	Sigma Life Science	E6758

Trizma®Base	TrisBase	Sigma Life Science	93350
Sodium hydroxide pellets	NaOH pellets	Sigma- Aldrich	06203
4-(2Hydroxyethyl)piperazine-1-ethanosufonic acid	HEPES	Sigma Life Science	H3375
Potassium Chloride	KCl	Riedel-de-Haen	31248
Bovine Serum Albumin	BSA	Sigma Life Science	A2153
Potassium Hydroxide	KOH	Sigma-Aldrich	60370
Hydrochloric acid (37%)	HCl	Riedel-de-Haen	30721
Hydrogen peroxide solution 30% solution in water	H_2O_2	Sigma Life Science	H1009
Formamidopyrimidine DNA glycosylase	FPG	A.Collin Laboratory	
Fetales bovine serum	FBS		
Dimethyl sulfoxide	DMSO	Sigma Life Science	D4540
t-octylphenoxypolyethoxyethanol	Triton X-100	Sigma Life Science	
Ethidium bromide stock solution (10 µg/ml)	EB	Sigma Life Science	E1510
Trypan blue solution		Invitrogen life technologies	T10282

4.4.2 Preparation of general reagents used for SCGE

Agarose

Normal melting agarose (NMA)

400 mg NMA

40 ml aqua bidest.

400 mg NMA were dissolved in 40 ml aqua bidest under heat treatment in a microwave.

Low melting agarose (LMA)

200 mg LMA

20 ml PBS

200 mg LMA were dissolved in 20 ml PBS under heat treatment in a microwave. Finally NMA solution was stored at 4°C in a cooling chamber before usage.

Lysis solution (pH=10)

Amounts per liter:

2.5 M NaCl	146.1g
0.1 M EDTA	29.2 g
10 mM TrisBase	1.211g

Additionally:

10 M NaOH	(approx. 40 ml/500 ml lysis solution)
Triton X-100	1ml/100 ml Lysis solution

First NaCl and TrisBase were mixed together and refilled with 500 ml aqua bidest. In the next step 20 ml NaOH and EDTA were added to the solution. Finally pH was adjusted to 10 with NaOH (approx. 20ml). Lysis solution was filled up until 1000 ml and stored in a dark bottle at 4°C in a cooling chamber.

Alkaline electrophoresis buffer (pH > 13)

Amounts per two liter:

0.3 M NaOH	24.0 g
0.001 M EDTA	0.58 g

First NaOH pellets were dissolved in aqua bidest and in the next step EDTA was added to the buffer. Finally pH was adjusted to pH 13 or higher. Previous to use, buffer was stored at 4°C in the cooling chamber.

Enzyme reaction buffer for FPG, stock solution (10x stock)

Amounts per liter:

40mM HEPES	95.3g
0.1M KCl	74.56g
0.5mM EDTA	1.46g
0.2mg/ml BSA	2g

Additionally: 1M KOH

HEPES, KCl, EDTA and BSA were dissolved in 500 ml aqua bidest and at the end the buffer was refilled up to 1000ml. For storage 20 tubes with 50 ml enzyme buffer were prepared. Aliquots were stored at -20°C. Previous to use, aliquots had to be defrosted and diluted to 1:10 with aqua bidest. In addition it was important to control the pH of the buffer and adjust it to 8 with KOH.

Neutralization buffer (pH=7.5)

Amounts per two liter:

0.4M TrisBase	96.88g
HCl 37%	approx. 50 ml

TrisBase was dissolved in 2000 ml aqua bidest and the pH was adjusted to 7.5 with 37% HCl. Buffer solution was stored in a dark bottle at 4°C in the cooling chamber.

H_2O_2 stock solution

H_2O_2 solution (30%)	103 µl
Aqua bidest	10 ml

To receive a 0.1M stock, 10,3 µl H_2O_2 solution (30%) were mixed with 1ml aqua bidest. H_2O_2 Stock solution was stored at 4°C in the cooling chamber.

Before use, the stock solution had to be diluted. For this purpose 80 µl of 0.1 stock were mixed with 79.9 ml aqua bidest.

10M NaOH solution

NaOH (40 g/mol)	400g
Aqua bidest.	1000 ml

400g NaOH were dissolved in 1000ml aqua bidest to receive a 10M NaOH solution.

1M KOH

KOH (56.11 g/mol)	56.11 g
Aqua bidest	1000 ml

56.11 g NaOH were dissolved in 1000 ml aqua bidest to receive a 1M KOH solution.

Ethidium bromide solution

Ethidium bromide stock	10 µl
Aqua bidest	5 ml

10µl Ethidium bromide stock was mixed with 5 ml aqua bidest to get a concentration of 20 µg/ml. Diluted Ethidium bromide solution was stored at 4°C.

4.4.3 Enzymes used to detect DNA oxidation damage

Detection of DNA damage provides limited information about DNA oxidation. DNA strand breaks and alkali labile sites (i.e. purinic/ apyrimidinic sites) can be a result from various forms of DNA damage and might also represent intermediates in the repair process. To achieve a higher level of sensitivity it is essential to implement a digestion step with a suitable enzyme that recognizes a particular kind of damage and induces a DNA strand break. The enzyme of choice is Formamidopyrimidine DNA glycosylase (FPG). FPG acts on 8-oxoGua and recognizes imidazole-ring-opened purines, or formamidopyrimidines. They appear during the spontaneous removal of damaged purines (COLLINS, 2004, 2009). *Instruction for FPG*: A final dilution of 1/1500 was used. Therefore 5µl of the FPG stock were mixed with 245 µl Enzyme reaction buffer- with the addition of 10% glycerol. 30µl aliquots were prepared and frozen at -80°C. For usage aliquots were diluted with Enzyme reaction buffer to the final concentration of 1/1500.

4.5 SINGLE CELL GEL ELECTROPHORESIS (COMET ASSAY)

The outlined protocol for the detection of oxidized bases with the SCGE (comet assay) is based on the reviews of A.Collins and Azqueta et al. (COLLINS, 2004; COLLINS et al., 2008; AZQUETA et al., 2009).

4.5.1 Slides preparation

The NMA was melted in a microwave until it was completely dissolved. The microscope slides were immediately covered with the hot NMA. Finally they were dried and stored at room temperature.

4.5.2 Isolation of peripheral blood mononucleated cells (PBMC´s)

Isolated PBMC‘s were used on control slides for alkaline electrophoresis. The same PBMC control stock was used for the first period of the study.

Procedure: whole blood was collected by venipuncture in heparin tubes. 6-7 ml of anticoagulant blood was transferred from heparin tube into a Leucosep tube. Leucosep tubes were spun at 16 °C at 1000 rcf/g for 15 minutes. All further steps were performed under cooling conditions. There are two further steps of centrifugation: Centrifugation step 1: 304 rcf/g, 15 minutes at 4°C, with brake and Centrifugation step 2: 304 rcf/g, 10 minutes at 4°C, with brake. Before the first centrifugation step the enriched fraction of PBMC‘s was transferred in another tube and filled up with cool PBS to 15 ml. After the first centrifugation step the supernatant was discarded and PBMC pellet was washed in 10 ml PBS buffer. After the second centrifugation step the supernatant was also discarded and PBMC pellet was suspended in 1 ml cool PBS (**Fig.9**). For the storage of PBMC´s aliquots, a suitable freezing solution was prepared containing: FBS, RPMI, and 10 %DMSO to preserve viability.

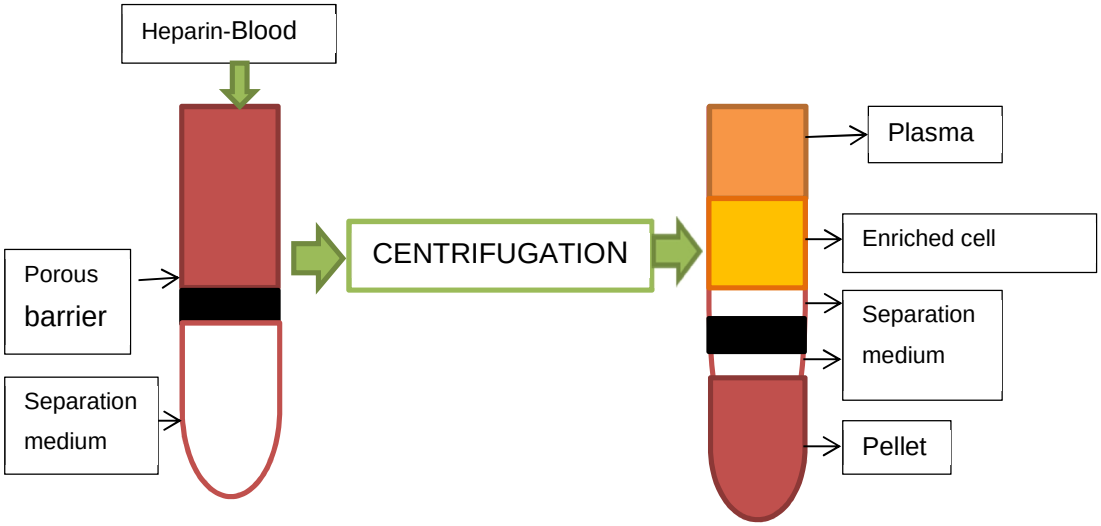

Figure 9: Isolation of peripheral blood mononucleated cells

4.5.3 Viability and PBMC‘ count

A high viability of PBMC´s is needed for the SCGE. There is a simple and sensitive method to test the viability of cells, the so called ‘trypan blue exclusion test’. Trypan blue was added to the cell suspension. Cells with a damaged membrane or dead cells incorporate the dye and appear blue (COLLINS, 2004; COLLINs et al., 2008). For this method we used the automated cell counter ‘Countess’ from Invitrogen. It was also used for determination of PBMC count in the cell suspension. To prevent overlapping comets and to insure adequate cell number per slide a precisely defined cell concentration of $1x10^6$ cells/ ml is needed (COLLINS, 2004). *Procedure:* 10µl trypan blue were added to 10 µl cell suspension. Finally 10µl of the mixture were transferred into a counting chamber slide.

4.5.4 Embedding cells in agarose

Slides covered with NMA were used. The labeling of the slides contained the subject number and the kind of treatment including lysis, H_2O_2, buffer and FPG. Slides were placed on cooled metal plates. The jar with LMA was placed in the water bath and heated up to 37°C.

30 µl cell suspension and 140 µl agarose were mixed. Immediately two drops of 70 µl of cell- agarose suspension were placed on the slide. Quickly the drops were covered with microscope cover glasses. The prepared slides were stored in the cooling chamber for 10 minutes.

All further steps including H_20_2 -, lysis treatment, alkaline electrophoresis and neutralization were performed in a cooling chamber at 4 °C.

4.5.5 Treatment with H_2O_2

For the treatment with hydrogen peroxide the slides were placed in a jar with 100µl diluted hydroxide peroxide solution. Incubation time was five minutes at 4°C. After incubation the slides were washed in PBS for another five minutes. Finally they were treated with lysis solution.

4.5.6 Treatment with lysis solution

The slides without the previous hydrogen peroxide treatment were immediately placed in jars with lysis solution. The slides with hydrogen peroxide treatment and the slides for enzyme treatment were placed in extra jars. The incubation time was one hour at 4°C.

4.5.7 Treatment with FPG and enzyme reaction buffer

Previous to the treatment with FPG the slides were washed three times for five minutes with enzyme reaction buffer (ERB). *Treatment with ERB:* 50µl ERB were applied to agarose gels and covered with microscope cover glasses (22x22). *Treatment with FPG:* 50µl FPG solution was put on the agarose gels and also covered with microscope cover glasses (22x22). The treated slides were incubated in a moist box at 37°C for thirty minutes.

4.5.8 Alkaline single-cell gel electrophoresis

Before starting with the electrophoresis it was important to incubate all prepared slides with alkaline electrophoresis buffer for twenty minutes at 4°C.

After incubation the electrophoresis program was started: 30 minutes at 25V and 300mA.

4.5.9 Neutralization procedure

Alkaline electrophoresis slides were washed in the first step in neutralization buffer (pH=7.5) for five minutes at 4°C and in the second step they were washed in aqua bidest for further five minutes at 4°C. The slides were dried at room temperature before staining.

4.6 STAINING WITH ETHIDIUM BROMIDE AND VISUALIZING COMETS

Dying of the gels occurred with 30 µl ethidium bromide solution (20 µg/ml). *Visualization of comets*: DNA comets are visualized by suitable fluorescence microscope. Stained gels had to be counted at the same day. *Principle:* 50 cells per gel were counted.

4.6.1 Evaluation of DNA oxidation- measuring comets

Komet 5.5 image analysis system was used for the analysis. This program detected the following parameters: DNA tail length, relative fluorescence intensity of head and tail. These parameters are normally expressed as percentage of DNA in the tail (% tail DNA).

4.7 STATISTICAL ANALYSIS

For statistical analysis SPSS 18.0 for Windows and Microsoft Office Excel 2010 were used. The data was expressed as mean ± standard deviation (SD).

Prior to statistical analysis (t-test) the results were tested with the Kolmogorov-Smirnov test of normal distribution. During the further analysis we used the paired two- sample t-test and regression analysis. The level of significance was defined as $p < 0.05$. The diagrams were compiled with SPSS 18.0 and Excel 2010.

5. RESULTS

5.1 STUDY COLLECTIVE

The preexamination (T_1) started in October 2011. 35 subjects participated in the first biochemical checkup (SCGE). First goal was to determine the status of DNA damage (DNA tail %) prior to the start of the interventional trial. After the first analysis subjects started their strength training under supervision.

In January 2012 the second period of biochemical analysis followed. Five subjects had to interrupt the participation in this trial because of health issues. Therefore we investigated the data of only 30 subjects (**Tab.11**). Only those subjects that completed both time points (T_1 and T_2) of the trial were used for the final statistical analysis.

Concurrent to the investigations of the second period (T_2), we also analyzed the first period (T_1) of the new subjects (n=42). This data set was not included in the analyses.

Table 11: Number of subjects at T_1

Intervention groups	Number of subjects
Control (cognitive group)	10
Training	11
Training & Nutrition	9

5.1.1 Age distribution

Subjects were on average 84,3 ± 6,04 years old. The youngest was 65 years and the oldest 98 years old. The largest group represents the participants above 85 years with 58%. Participants between 64-75 years represent the smallest group (**Fig.10**).

The age distribution in our trial is consistent with the expectancy of life of women and men in Austria. At present the expectancy of life is 83,3 years for women and 78,0 years for men (STATISTIK AUSTRIA, 2013).

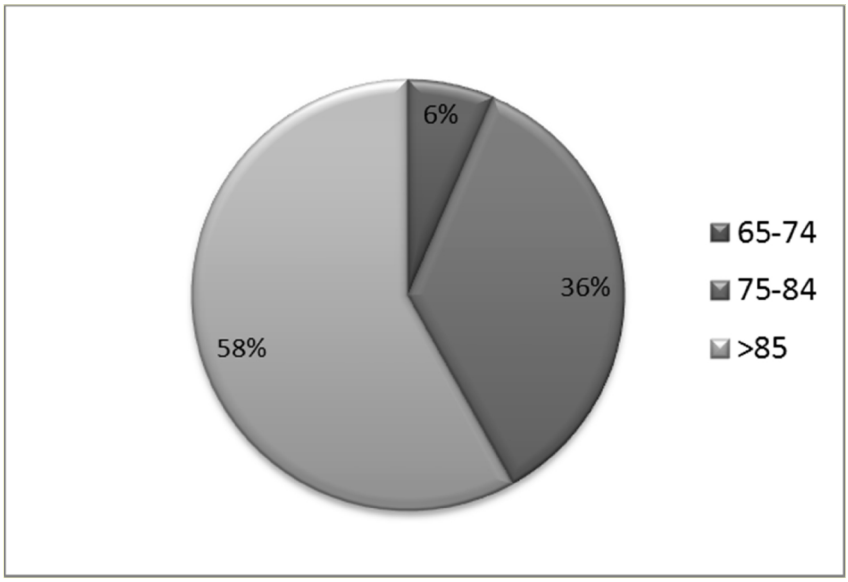

Figure 10: Age distribution of the subjects N=30

We surveyed the age distribution in the different intervention groups and concluded that subjects between 75-84 years were overrepresented in the training group compared to both other groups at that time point of the recruitment. In the control group the majority was represented by elderly above 85 years.

Table 12: Mean age of subjects

Intervention group	Mean age
Control	87,0 ± 4,86
Training	83,09 ± 3,82
Training & Nutrition	82,89 ± 8,10

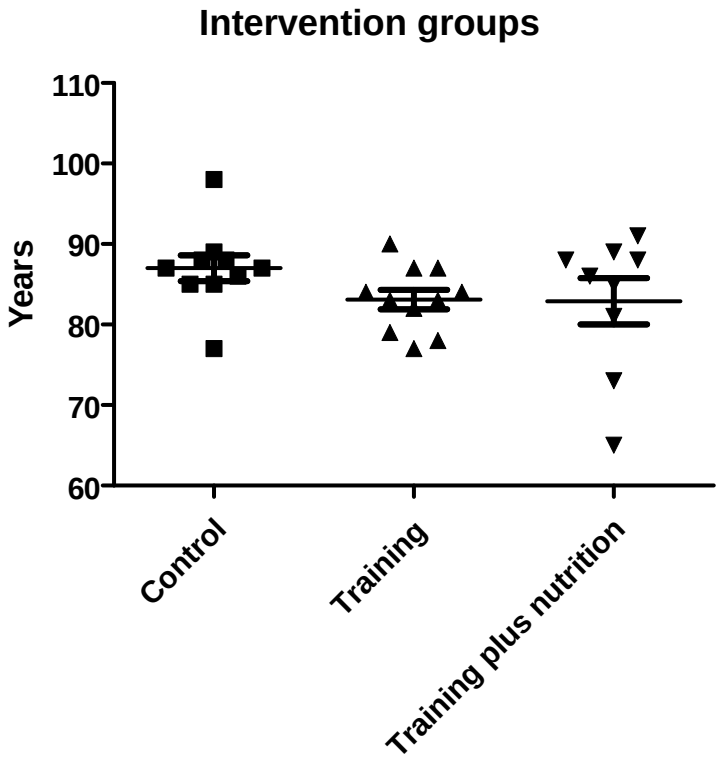

Figure 11: Age distribution between groups

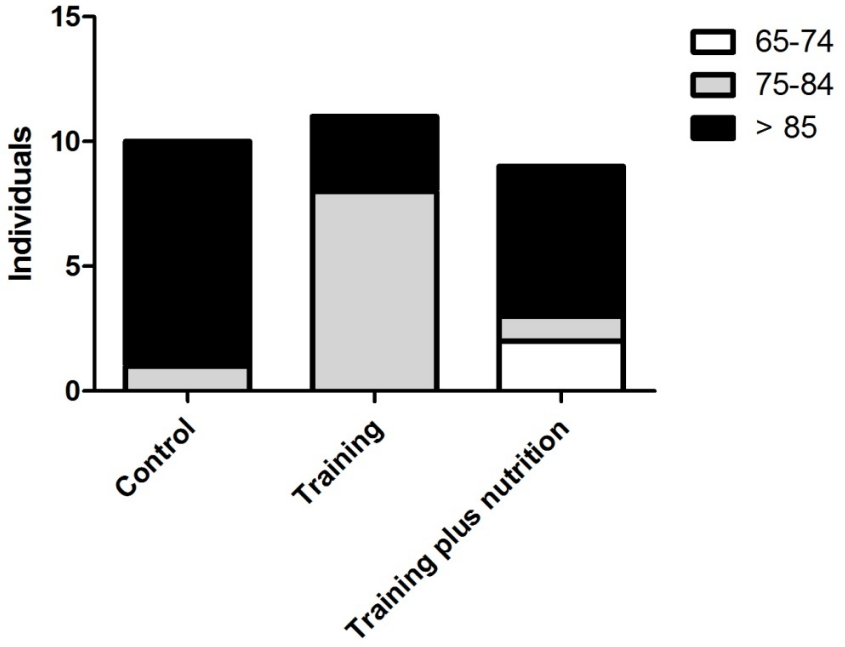

Figure 12: Age distribution in groups

5.1.2 Sex distribution

The life expectancy of women with 83,3 years is on average five years higher than the life expectancy of men with 78,0 years (STATISTIK AUSTRIA, 2013). The mean age in the trial was 84,3 ± 6,04 years. Women represent with 74% the higher amount of participants (**Fig. 13**).

In particular in the intervention groups women represent the majority, especially in the training & nutrition group with around 80%.

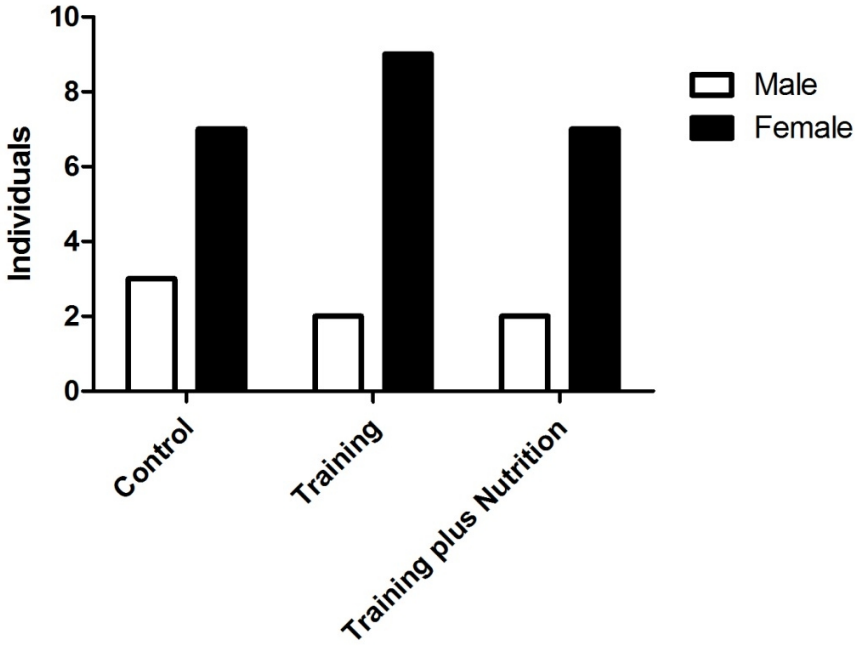

Figure 13: Sex distribution between intervention groups

5.1.3 Body Mass Index

The Body Mass Index (BMI) is a frequently used method to classify the individual's weight into: normal weight, underweight, overweight and obese (**Tab.13**).

Table 13: Body Mass Index for adults; BMI= body weight / body height 2 (kg/m^2) (DGE, 2013)

Body weight	BMI (kg/m$^{2)}$
Under weight	< 18,5
Normal weight	18,5-24,9
Overweight	25,0-29,9
Obesity	>30,0

The BMI is a suitable method to classify the body weight and to predict a possible risk for cardiovascular diseases. For the elderly it is not the method of choice, because of age related body height alterations and alterations in body fat distribution. In adults an increase in BMI is associated with higher risk for cardiovascular diseases. In the elderly a slight increase in BMI is associated with a reduced mortality. A normal BMI for elderly (above 65 years) is 24,0-29,9 kg/m^2 (BMG, 2013). In the present nutrition report of Austria the mean BMI for elderly women is 28,8 kg/m^2 and for men 27,9 kg/m^2.

Participants in our trial showed mean BMI of 28, 21 ± 4, 74 kg/m^2. The mean BMI for women is 28,89 ± 4,96 kg/m^2 and for men 26, 26 ± 2,88 kg/m^2. This pretty much is in agreement with the data from the nutritional report. We assessed the BMI at both periods T_1 and T_2. After three month intervention there are only marginal changes in the BMI. There is only a little increase in women´s BMI. However it was not significant. We could not assess differences of BMI in the different intervention groups.

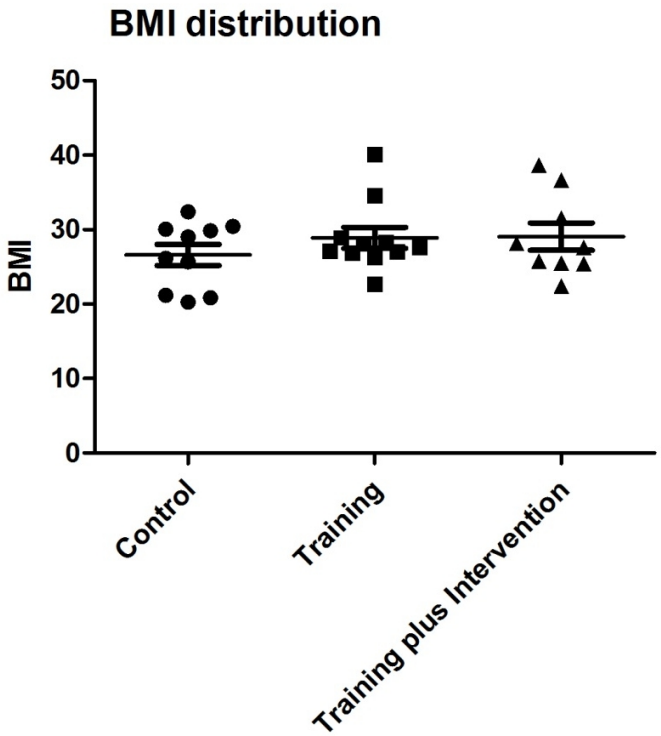

Figure 14: Mean BMI of subjects (period T_1)

5.2 RESULTS FROM SINGLE CELL GEL ELECTROPHORESIS (SCGE)

For the assessment of results we used the percentage of tail DNA. These data were obtained after cell counting. Data of interest are the arithmetic mean of tail DNA. For statistical analysis between the particular intervention groups we used the paired two- sample t-test for dependent data. Furthermore we also conducted the paired two-sample t-test for independent data.

5.2.1 Effect of three months training on double strand breaks

5.2.1.1 Results from paired two sample t-test for dependent samples

In the training group we observed significant reduction of oxidative DNA damage after H_2O_2 treatment. Furthermore we saw a significant reduction of oxidative DNA damage in the training plus nutrition group, also after H_2O_2 treatment.

In the control group there was no significant change in DNA damage.

Table 14: T_1 /T_2 comparison H_2O_2 Treatment

Group	N	Mean T_1	Mean T_2	p-value
Control	10	24,73 ± 5,29	20,02 ± 9,21	0,159
Training	11	27,16 ± 7,56	15,73 ± 10,07	**0,015**
Training& Nutrition	9	27,72 ± 10,24	20,64 ± 8.07	**0,026**

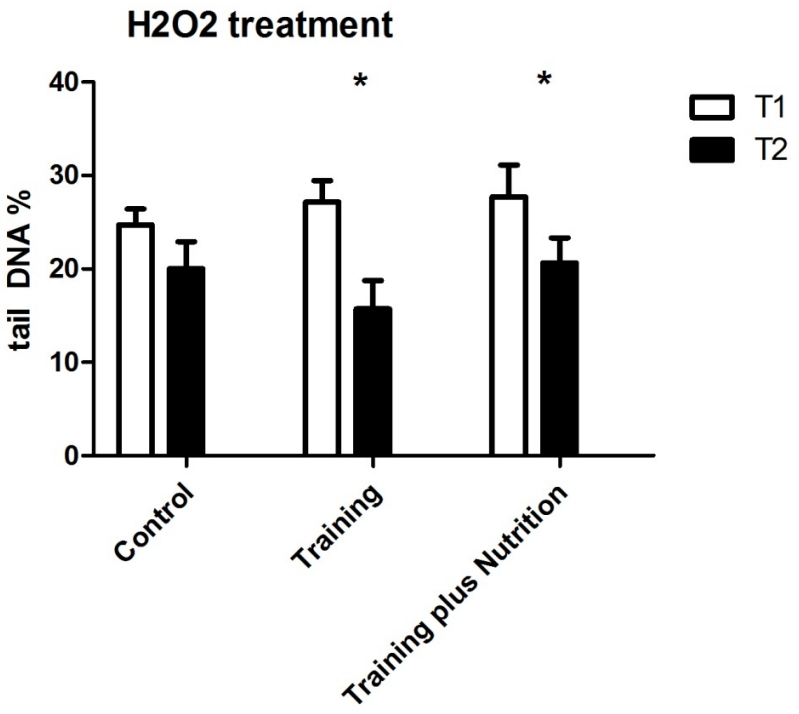

Figure 15: Tail DNA% after H_2O_2 treatment

Furthermore we analyzed our results for H_2O_2 treatment regarding gender aspects. Also after excluding male participants we see a significant reduction in tail DNA, in both the training only and training plus nutrition groups. This proves that also female individuals above the age of 65 years profit by 3 months of physical activity.

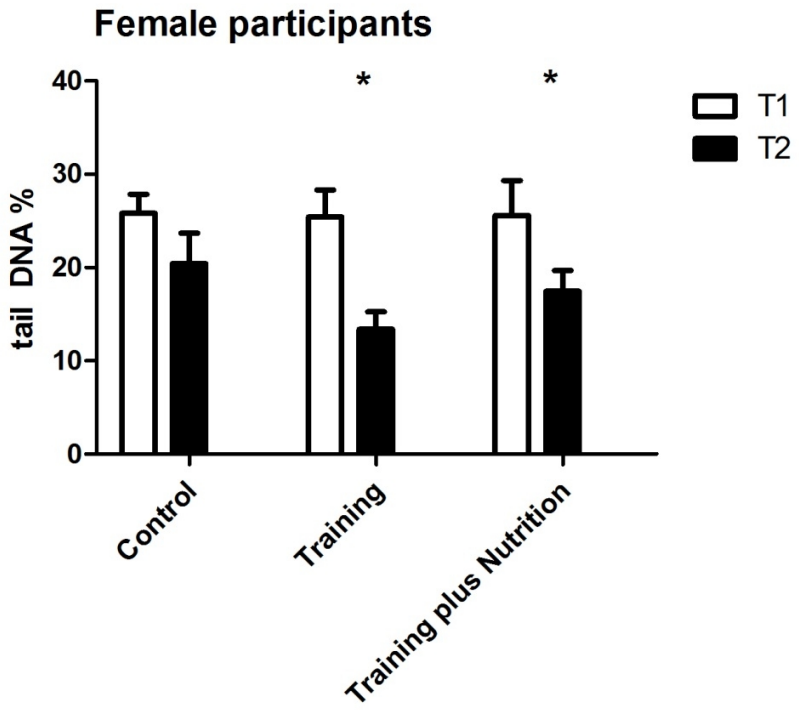

Figure 16: Tail DNA % after H2O2 treatment in female participants

Under lysis treatment, as well as FPG treatment we were not able to see significant changes in the level of DNA strand breaks in the training and training plus nutrition groups. But also here a trend towards reduction of measured tail DNA is observable. The lack of significance might be because of high variability of the results, leading to high standard deviations.

Table 15: T1 /T2 comparison Lysis Treatment

Group	N	Mean T1	Mean T2	p-value
Control	10	6,01 ± 1,63	7,53 ± 2,03	0,143
Training	11	6,52 ± 3,79	6,71 ± 1,37	0,805
Training & Nutrition	9	7,67 ± 3,03	6,67 ± 1,37	0,490

Table 16: T1 /T2 comparison FPG Treatment

Group	N	Mean T1	Mean T2	p-value
Control	10	11,01 ± 7,50	10,31 ± 4,63	0,824
Training	11	11,85 ± 12,90	12,18 ± 9,74	0,706
Training & Nutrition	9	12,13 ± 13,88	7,61 ± 6,74	0,436

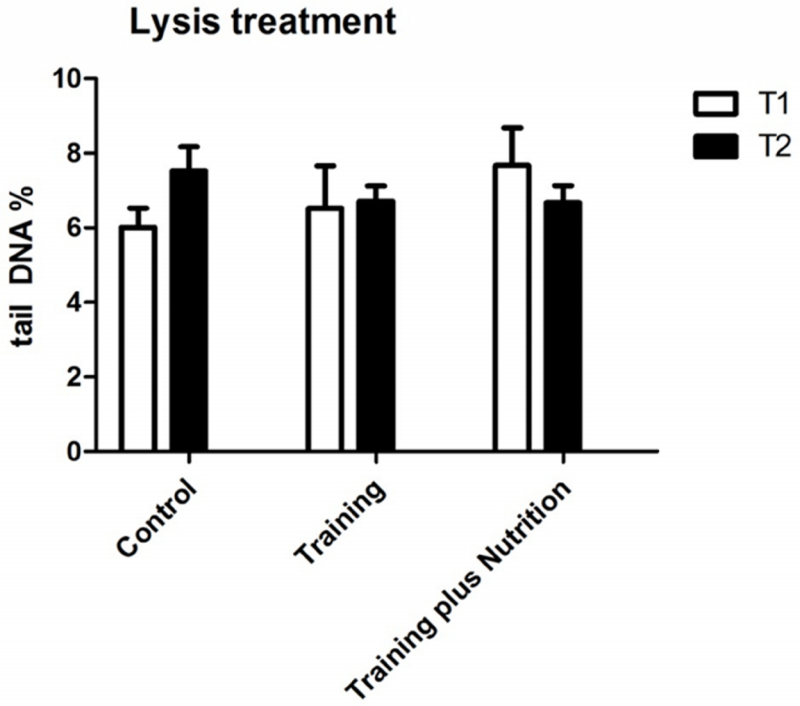

Figure 17: Tail DNA % after Lysis treatment

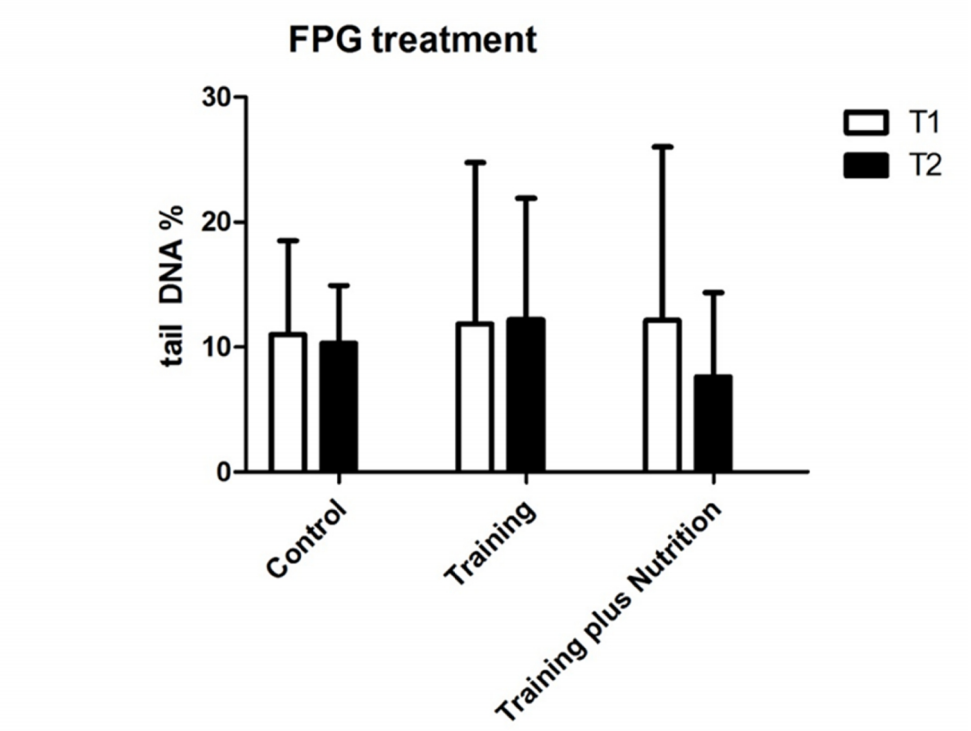

Figure 18: Tail DNA % after FPG treatment

5.2.1.2 Results from regression analysis for age groups

We also wanted to know if reduction of oxidative DNA damage is age-dependent. The hypothesis was that no matter how old our participants are, all of them will have a reduction in DNA damage by the same extent. So we performed a regression analysis by plotting on one axis the age of our participants and on the other axis the reduction in tail DNA in percent (delta tail DNA %). We were not able to see any correlation between age and change in tail DNA, neither in the control group nor in the interventional groups. This proofs the fact that physical activity is able to reduce DNA damage independent of age.

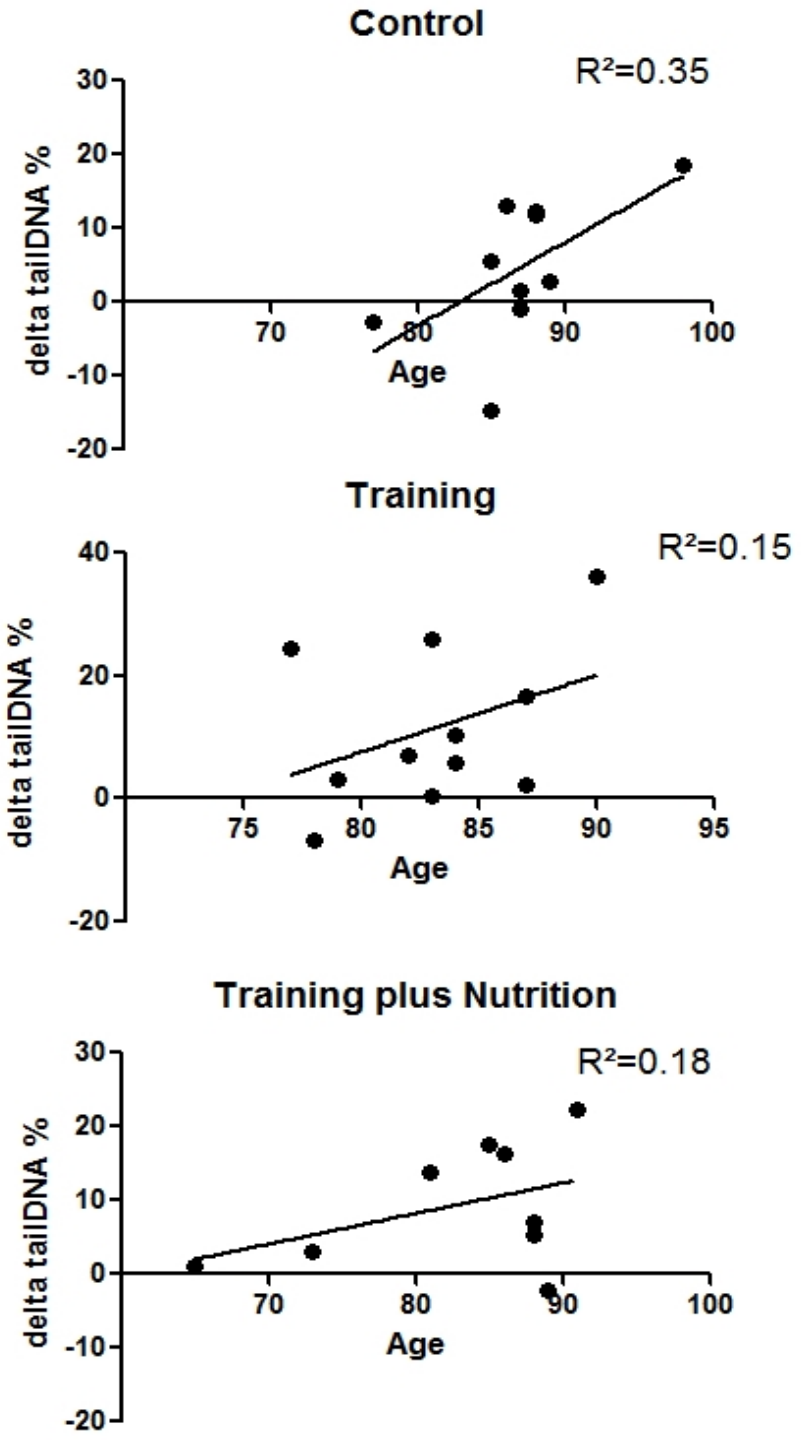

Figure 19: Regression analysis for age groups

6. DISCUSSION

6.1 DISCUSSION OF INTERVENTION METHOD

After the first three months of intervention we see a tendency towards reduction of oxidative DNA damage in elderly people in the training group. In the training plus nutrition group we were able to proof a significant reduction of DNA double strand breaks, as measured by SCGE.

This seems to be in line with a study performed by Traustadóttir et al. that showed that physically fit older adults had a significantly lower level of oxidative stress than unfit age-matched controls (TRAUSTADÓTTIR et al., 2012). The study mentioned above was no interventional trial, but a cross-sectional comparison between fit and unfit older men and women. They only measured the fitness levels of the participants by means of maximal leg power and VO2max (performed on a cycling ergometer). Levels of oxidative stress were measured by urinary markers of nucleic acid damage and lipid peroxidation. The antioxidant status was assessed by measuring the ratio reduced to oxidized glutathione in plasma. Due to the study design of our trial (randomized, interventional) the results should be more reliable.

On the other hand Cash et al. performed a very similar trial to the one presented in this thesis. 220 healthy participants between the ages of 50-76 underwent exercises at different intensity levels. Comparable to our study the authors were not able to see any association of even high-intensity training with an increase in DNA damage. In fact any measure of activity led to better DNA repair mechanisms also measured by the comet assay (CASH et al., 2013).

The same results have also been shown in sedentary obese men. Interestingly obese subjects had at baseline higher levels of oxidative damage. Those participants underwent a 3 month moderate-intensity cycling training. In agreement with our study, endurance exercises reduced the levels of ROS in obese patient.

Besides training also reduced the level of insulin resistance as well as led to an improvement in cytokine profiles which are associated with obesity (SAMJOO et al., 2013).

6.2 DISCUSSION OF LYSIS, H_2O_2 AND FPG TREATMENT

6.2.1 Lysis Treatment

In a review Hoelzl et al. reported that lysis treatment of cells leads to a reduction of about one third in DNA-migration capability. Lysis treatment is only able to detect single or double strand breaks (HOELZL et al., 2009). That's why lysis treatment is less sensitive and therefore less significant than H2O2 or FPG-treatment. H2O2 is an exogenous ROS and damages vital cellular structures, in particular those of the mitochondria. Resistance to H2O2 treatment represents the capability of endogenously produced anti-oxidant proteins, such as heat-shock proteins, to counteract oxidative damage. FPG on the other hand is an enzyme that induces the repair of oxidized pyrimidines and purines by excising them and cutting the backbone of the DNA. Thereby additional strand breaks are induced at the location of oxidized base, making this assay way more sensitive than lysis treatment on its own. One has to discuss that this might be the reason why after lysis treatment no significant reduction in DNA damage after 3 months of intervention was found.

We saw that at the baseline oxidative DNA damage was by trend higher in the training and training plus nutrition group when compared to the cognitive training group. However statistically it is not significant. This implicates that already at the beginning of the study DNA damage was higher in both intervention groups. After three months of intervention we couldn´t detect significant changes between the groups.

6.2.2 H_2O_2 Treatment

As mentioned above, we saw a significant reduction of oxidative DNA damage in the training and training plus nutrition group. Concluding from this observation one can hypothesize that physical activity leads to an increase in resistance towards oxidative damage. In a trial performed by Cash et al. oxidative damage was provoked by gamma radiation rather than H2O2 treatment. Repair capacity of cells was measured after 15 and 60min after irradiation. Cells from individuals that performed high-intensity training were more likely to have increased repair mechanisms after 60 minutes of irradiation. The authors were also able to show that each additional hour of physical activity per weak increased mean DNA repair capacity by 0.21% (CASH et al., 2013).

In a different trial Siu et al. used rats to examine the effects of 8 and 20 weeks of physical activity on oxidative DNA damage and antioxidant enzyme activities, for instance superoxide dismutase or glutathione peroxidase. 40 female rats were assigned to 4 different groups. There was a sedentary control and an exercise group for an experimental period of 8 or 20 weeks. Rats in the interventional group had 24h access to an in-cage running wheel. On the opposite rats in the control group were kept without a running wheel. After 8 as well as 20 weeks of intervention, rats kept with a running wheel showed a significant reduction by 21 and 45% in DNA damage respectively, as measured by comet assay (**Fig.20**). Interestingly also the expression of antioxidant enzymes like superoxide dismutase or glutathione peroxidase was significantly elevated. This verifies that habitual exercise protects cells from oxidative damage (SIU et al., 2011).

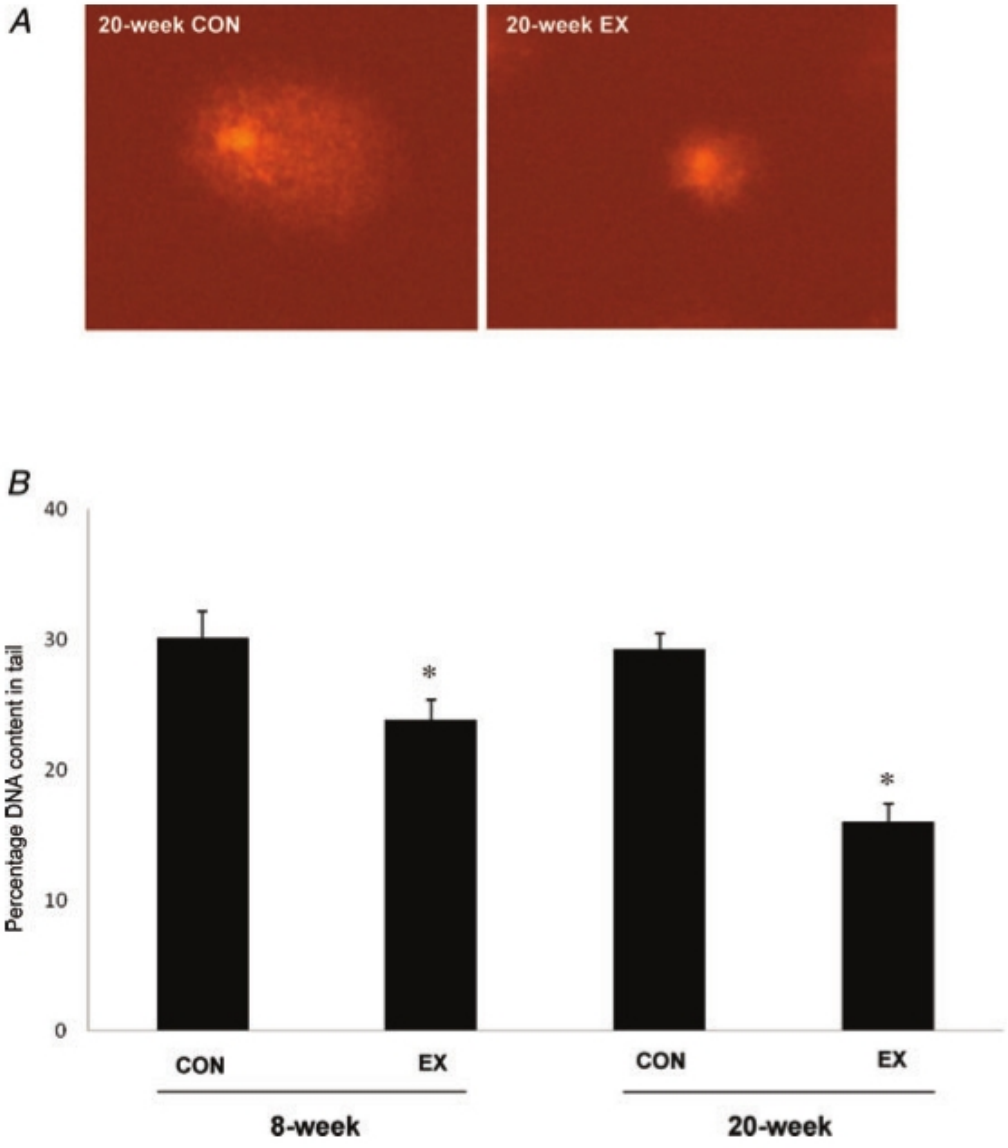

Figure 20: Reduction of oxidant-induced DNA damage (SIU et al., 2011)

The results of the studies above are pretty much in agreement with our interventional trial. It is unclear if the supplementation of FortiFit in the training plus nutrition group influenced DNA damage. FortiFit was designed to influence biosynthesis of muscle proteins positively to counteract sarcopenia in the elderly. Furthermore the supplement contains antioxidative vitamins, which could have a positive effect on DNA damage. In our trial reduction in DNA damage after H_2O_2 treatment is comparable in the training group and training plus nutrition group. So one has to assume that most of the effect can be traced back to physical activity, but it cannot be ruled out that nutritional supplementation also had possible effects on DNA stability.

6.2.3 FPG Treatment

8-Hydroxydeoxyguanosine (8-OHdG) is the main ROS product that can induce mutations (BARZILAI and YAMAMOTO, 2004). 8-OHdG is an oxidized guanine and is therefore misincorporated during DNA synthesis. Such DNA damage cannot be detected by standard lysis treatment. Therefore one has to use a lesion-specific enzyme called formamidopyrimidine DNA-glycosylase (FPG). In cells treated with FPG the major purine oxidation product 8-oxoguanine is converted into a double strand break of DNA that can be measured by a regular comet assay (HOELZL et al., 2009; MOTA et al., 2010).

After three months of intervention we saw no significant result when samples were analyzed after FPG treatment. We are only able to detect a tendency towards reduction of DNA damage in the training plus nutrition group. Unfortunately we observed a high level of heterogeneity of the test results. This led to high standard deviations that distort the actual results. One explanation might be a reduced activity of the FPG enzyme during our analysis.

In a similar study performed by Mota et al., the influence of aerobic fitness on age-related lymphocyte DNA damage in humans was examined. The authors were able to show that DNA damage induced by oxidative stress in FPG sites was more

prominent in the age group of 40 years and older than in younger individuals (19-29 and 30-19 years old). Moreover subjects with a lower baseline aerobic fitness level had a higher degree of DNA damage, independent of age (MOTA et al., 2010).

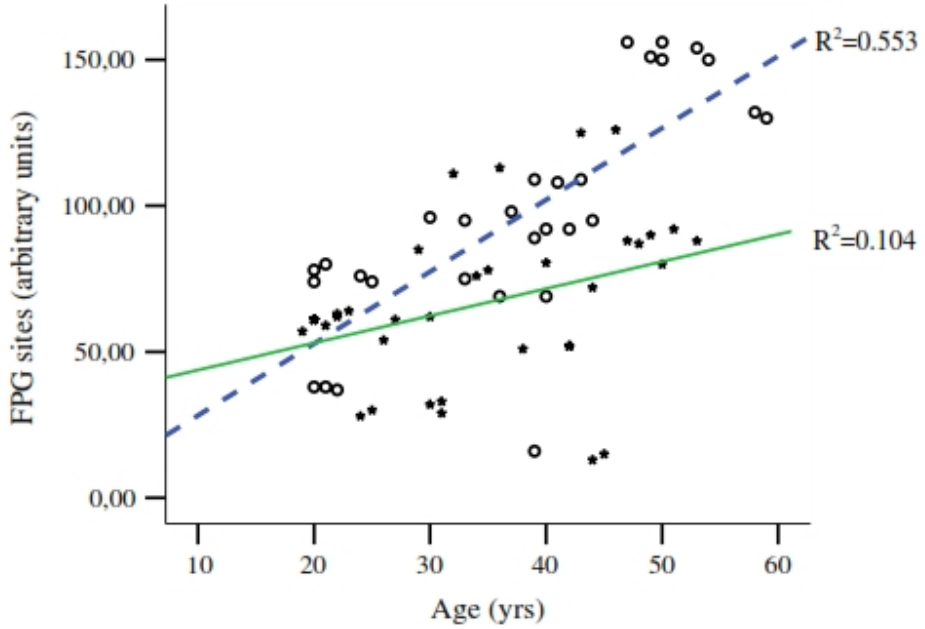

Figure 21: Linear regression between FPG sites and age (low fitness= blue, broken lines; high fitness= green, solid lines) (MOTA et al., 2010)

In a different study performed by Soares et al. DNA damage in human lymphocytes was correlated with age und VO2max, as a measure of maximum oxygen uptake. The latter parameter reflects aerobic physical fitness of an individual. The trial was performed with 36 male, healthy, non-smokers aged 20-84 years. They found a positive correlation between age and DNA strand breaks. Interestingly the results were not significant for FPG-sensitive sites (SOARES et al., 2013).

This is in agreement with our study. We were also not able to see differences in DNA damage after training in FPG sensitive sites. Contrary to that we had significant results for H2O2 induced DNA damage.

7. CONCLUSION

The main question in this study to answer was if a three month period of strength training and dietary intervention with a supplement (FortiFit) is able to reduce oxidative DNA damage in a collective of people at the age of 65 years and above.

After 3 months we were able to demonstrate a significant reduction of oxidative DNA damage, measured by comet assay and H2O2 treatment of cells, in the training and the training plus nutrition group.

This proofs the fact that lymphocytes from patients that underwent physical training acquired a certain kind of resistance against reactive oxygen species. Particularly one has to mention that we were able to proof that this effect is independent of age and gender. In detail we showed that even the oldest of our participants profit from physical training and that female as well as male individuals show a reduction in DNA damage.

Samples treated with lysis buffer only or FPG did not show significant results. This most probably can be traced back to high standard deviations of our measurements and a reduced activity of FPG enzyme.

Our results confirm existing studies showing an increased resistance of cells against oxidative DNA damage after moderate and periodic strength training.

In the future it will be interesting to see if our results will be verified or even strengthened in the still ongoing study. Possibly the data is strong enough to publish recommendations for strength training in geriatric patients.

8. SUMMARY

This paper is part of a one and a half years human interventional trial of the Department of Nutritional Sciences and the Centre for Sport Sciences of the University of Vienna. The aim of this thesis is to answer the question if 3 month of guided strength training and nutritional intervention by supplementation are able to reduce oxidative DNA damage in participants at the age of 65 years and above. **Materials and Methods** 30 participants were randomized to 3 intervention groups, one training only group, one training plus dietary intervention group and a control group that performed cognitive training. Individuals assigned to one of the two training groups performed a 50 minutes moderate strength training twice weekly. Execution of the training complies with published guidelines of the American College of Sports Medicine. Another group not only underwent strength training but also received a supplement called FortiFit, designed against sarcopenia. After 3 months peripheral blood mononucleated cells of the participants were collected and subjected to 3 different treatments, being lysis buffer, H2O2- and FPG treatment. Comet assay was consecutively used to detect single- and double strand breaks as a result of oxidative DNA damage.

Results After 3 months of intervention we were able to detect a significant reduction of DNA damage in the training (p-value H_2O_2= 0,015) and training plus nutrition (p-value H_2O_2 = 0,026) intervention group as measured by comet assay. We were also able to show that this effect is independent of age and gender of our participants. Nutritional supplementation did not significantly alter oxidative DNA damage.

Conclusion Our results are consistent with published data about strength training and its effect on oxidative DNA damage. We were not able to show that nutritional supplementation with amino acids targeting sarcopenia had any additional beneficial effects on DNA integrity. More studies need to be performed in future to nail down the molecular pathways that promote DNA integrity after physical intervention in the elderly.

9. ZUSAMMENFASSUNG

Die vorliegende Arbeit ist ein Teil einer über eineinhalb Jahre angelegten humanen Interventionsstudie des Departments für Ernährungswissenschaften und des Zentrums für Sportwissenschaften der Universität Wien. In der vorliegenden Arbeit soll die Fragestellung geklärt werden, ob eine Intervention mit Krafttraining und Krafttraining und Ernährung (FortiFit Supplement) über drei Monate einen Einfluss auf die oxidative DNA Schädigung bei Hochbetagten (≥65 Jahre) hat.

Methode Die Daten von insgesamt 30 Probanden wurden ausgewertet. Alle Teilnehmer wurden zufällig einer Interventionsgruppe (Training, Training & Ernährung und Kontrollgruppe) zugeteilt. Die Teilnehmer der Trainingsgruppe absolvierten zwei Mal die Woche ein 50-minütiges Krafttraining. Die Durchführung des Trainings richtete sich nach dem Protokoll des American College of Sports Medicine. Die Training & Ernährungsgruppe erhielt zusätzlich zum Krafttraining ein Supplement (FortiFit) in Form einer Trinklösung. Die Kontrollgruppe wurde einem kognitiven Training unterzogen. Nach drei Monaten wurden den Probanden Blut abgenommen. Daraus wurden die PBMC´s (peripheral blood mononucleated cells) isoliert. Diese wurden drei verschiedenen Behandlungen unterzogen (Lysis, H2O2 und FPG Behandlung). Mittels SCGE konnte der Grad der DNA-Migration, als Folge von Einzel-und Doppelstrangbrüchen und somit auch die oxidative DNA Schädigung ermittelt werden.

Ergebnisse Nach drei Monaten konnte eine signifikante Reduzierung der oxidativen DNA Schädigung in der Trainingsgruppe (p-Wert H_2O_2 = 0,015) und der Training & Ernährungsgruppe (p-Wert H_2O_2 = 0,026) gezeigt werden. Dies war jedoch nur bei der Behandlung mit H2O2 signifikant. Diese Ergebnisse zeigen, dass die Lymphozyten nach drei Monaten Intervention eine gewisse Resistenz gegenüber ROS (H2O2) entwickelt haben und somit nicht mehr so anfällig für oxidative DNA Schädigung sind.

Schlussfolgerung Unsere Ergebnisse sind vereinbar mit der aktuell vorliegenden Studienlage bezüglich Training und oxidativer DNA Schädigung. Es konnte kein Benefit hinsichtlich DNA-Schädigung in jener Studiengruppe gefunden werden, die zusätzlich zum Training noch Nahrungssupplemente erhielten. Es werden weitere Studien von Nöten sein, um jene molekularen Signalwege zu identifizieren, die die positiven Effekte eines körperlichen Trainings auf die DNA-Integrität hervorrufen.

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11. CURRICULUM VITAE

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