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Influence of an Orally Effective SOD on Hyperbaric Oxygen-related Cell Damage

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In a prospective, double-blind, randomised placebo-controlled study, we tested the hypothesis that a new formulation consisting of wheat gliadin chemically combined with a vegetal (thus orally effective) preparation of superoxide dismutase (SOD) allows to prevent hyperbaric oxygen (HBO)-induced oxidative cell stress.

Twenty healthy volunteers were exposed to 100% oxygen breathing at 2.5 ATA for a total of 60 min. DNA strand breaks (tail moments) were determined using the alkaline version of the comet assay. Whole blood concentrations of reduced (GSH) and oxidised (GSSG) glutathione and F_2-isoprostanes, SOD, glutathione peroxidase (GPx) and catalase (Cat) activities and red cell malondialdehyde (MDA) content were determined.

After HBO exposure the tail moment ($p = 0.03$) and isoprostane levels ($p = 0.049$) were significantly lower in the group that received the vegetal formulation. Neither SOD and Cat nor GSH and GSSG were significantly affected by this preparation or HBO exposure. By contrast, blood GPx activity, which tended to be lower in the SOD-group already before the HBO exposure ($p = 0.076$), was significantly lower afterwards ($p = 0.045$).

We conclude that an orally effective SOD–wheat gliadin mixture is able to protect against DNA damage, which coincided with reduced blood isoprostane levels, and may therefore be used as an antioxidant.

Keywords: Comet assay; Isoprostranes; Glutathione; Malondialdehyde; DNA strand breaks; Antioxidants

INTRODUCTION

Under physiological conditions, the excessive production of reactive oxygen and nitrogen species, which are involved in numerous physiological and pathological pathways, is controlled by the antioxidant defences. Previous studies showed that hyperbaric oxygen (HBO) treatment may serve as an *in vivo* model for the investigation of oxidative stress in humans:[1] subjects exposed to HBO show increased blood levels of free radicals[2] and DNA damage as measured in leukocytes with the comet assay.[1] So far this DNA damage cannot be prevented by oral antioxidants such as Vitamin E or N-acetylcysteine.[3]

Although various antioxidant substances, such as vitamins, trace-elements or other nutritional compounds (i.e. flavonoids) are effective when administered orally, until recently efficient oral delivery of antioxidant enzymes has been limited by the gastro-intestinal digestive processes.[4] A new nutritional formula (Glisodin®) containing a plant (*Cucumis melo* LC) superoxide dismutase (SOD) extract chemically combined with a gliadin biopolymer system allows overcoming this limitation.[5,6]

In a prospective, double-blind randomised placebo-controlled study with healthy volunteers, we therefore tested the hypothesis that an orally effective mixture of the antioxidant enzyme SOD with gliadin allows preventing DNA strand-breaks affiliated with exposure to HBO.

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MATERIALS AND METHODS

Subject and Protocol

After approval by the ethical committee of the Ulm University Medical School and detailed explanation, 20 healthy male volunteers gave their written informed consent to participate in the investigation, which was conducted according to the principles of the "Declaration of Helsinki". Due to technical problems with the gel electrophoresis, data for the comet assay are available only for 17 of the 20 subjects (placebo-group $n = 9$, Glisodin®-group $n = 8$). All subjects were well experienced and trained scuba divers (Table I), on a normal diet without any supplementation with vitamins or chronic intake of medication, and were advised not to dive in the period of 2 weeks prior to HBO exposure. The administration of either the orally effective SOD–wheat gliadin mixture or the corresponding vehicle started after blinding and randomisation 14 days prior to HBO-treatment. The verum group had an intake of 1000 UI-NBT of SOD per day administered once a day in two capsules of Glisodin®. Glisodin® is a water soluble form of SOD-calibrated extract from *C. melo* LC chemically combined with wheat gliadin. This melon extract also contains other antioxidant components, but heat inactivation of the SOD activity nearly completely blunted the antioxidant effect in mice fed over 28 days.[7] The placebo group received two capsules daily containing the same amount of wheat gliadin alone. After spray drying and using maltodextrine as support, a powder is obtained containing one UI-NBT/mg, as assayed after extraction and gel electrophoresis separation by a specific enzymatic assay.[8,9] The subjects were exposed to HBO according to a routine treatment protocol consisting of 100% oxygen breathing at a pressure of 2.5 ATA for a total of 2×30 min periods, interspersed with a period of 10 min of air breathing. Venous blood samples were taken before HBO exposure and immediately on exit from the chamber. Blood samples for the comet assay were processed immediately on site, the others stored on ice and processed within 1 h.

Measurements

The alkaline comet assay was performed on whole blood, as described by Speit *et al.*[10] The cells were denaturated with alkali (pH 13) for 30 min and electrophoresis was performed for 25 min at 30 V and 300 mA. Measurements were made by image analysis (Comet Assay II, Perceptive Instruments, Haverhill, UK), determining the mean tail moment of 50 cells per slide.

The concentrations of whole blood reduced and oxidized glutathione (GSH and GSSG) were determined by use of a commercially available colorimetric determination kit (Bioxytech® GSH-GSSG-412™, OxisResearch, Portland, OR). Plasma 8-isoprostane (8-epi Prostaglandin F_2) levels, a direct marker of lipid peroxidation,[11] were determined by use of a 8-isoprostane enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI, USA).

Whole blood antioxidant enzymes were assessed as SOD (RANSOD kit, Randox Laboratories Ltd, UK) and glutathione peroxidase (GPx) (RANSEL kit, Randox Laboratories Ltd, UK). Catalase (Cat) activity was assayed by a method in which the disappearance of peroxide is followed spectrophotometrically at 240 nm as described by Aebi.[12]

Red cell malondialdehyde (MDA) content was determined using the reaction of MDA with diethylthiobarbituric acid.[13]

Statistical Analysis

Because of exclusion of normal distribution using the Kolmogorov–Smirnoff-test all data are presented as median (quartiles) unless otherwise stated. Differences within the groups, e.g. before vs after HBO exposure, were analysed using a paired Wilcoxon rank-sum test. Differences between the placebo and the verum group were analysed using a Mann–Whitney rank-sum test for unpaired test samples.

RESULTS

Table I shows the demographic and anthropometric data of the volunteers. No significant difference was found with respect to age, height, weight or diving experience and current diving activity.

Figure 1 shows the results of the comet assay. There was no intergroup difference prior to the HBO exposure: tail moment was 0.34 (0.25–0.43) and 0.26 (0.23–0.34) for the placebo and Glisodin® groups, respectively. While the tail moment slightly increased in the placebo group from 0.34 (0.25–0.43) to 0.42 (0.33–0.53) ($p = 0.098$), no change

TABLE I Demographic and anthropometric data of the subjects

Median (quartiles)	Number (n)	Age (years)	Weight (kg)	Height (cm)	Diving experience (years)	Dives per year
Placebo	10	33.5 (28.25–44)	82.5 (72–88.75)	177.5 (173–185.75)	9 (6.25–13.75)	87.5 (22.5–100)
Glisodine	10	28 (28–36.5)	86 (76–91.5)	181.5 (174.5–186.5)	9.5 (6.5–14.75)	37.5 (30–48)

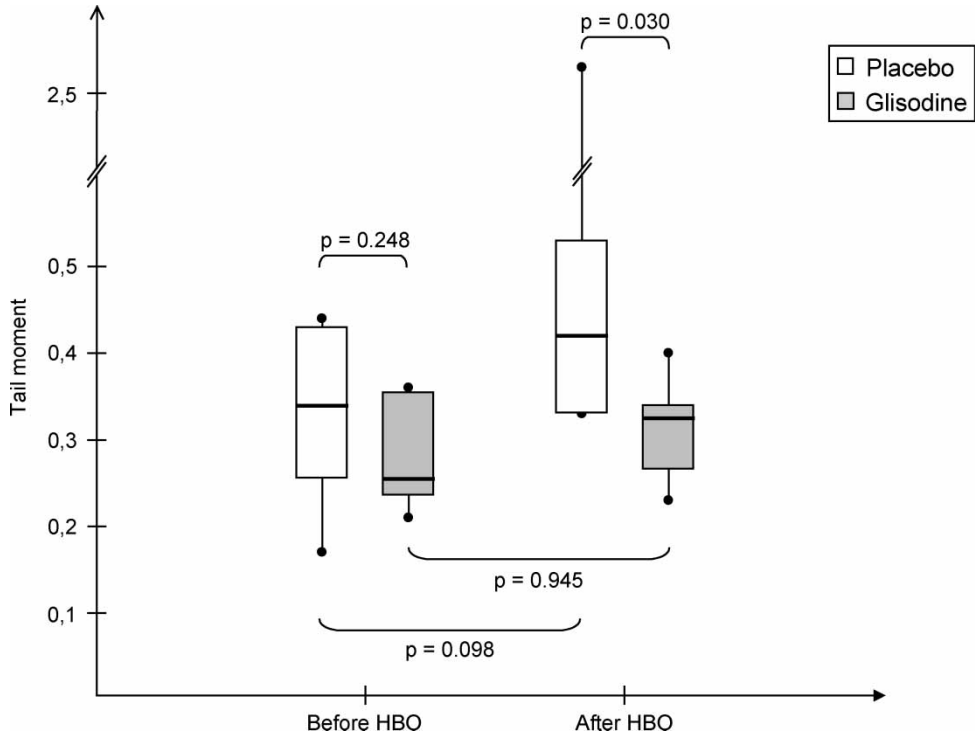

FIGURE 1 *Results of the comet assay.* Data are presented as boxplots displaying median, quartiles and extremes. Open boxplots represent the data of the placebo-group ($n = 9$), grey boxplots represent the data of the Glisodin® group ($n = 8$).

was observed in the Glisodin® group from 0.26 (0.23–0.34) to 0.32 (0.26–0.34), which resulted in a significant difference ($p = 0.03$) between the two groups after HBO.

Figure 2 demonstrates that the plasma isoprostane levels responded comparably: while there was no intergroup difference in the baseline values (16.8 (12.4–23.5) pg/ml vs 14.0 (12.1–19.9) pg/ml in

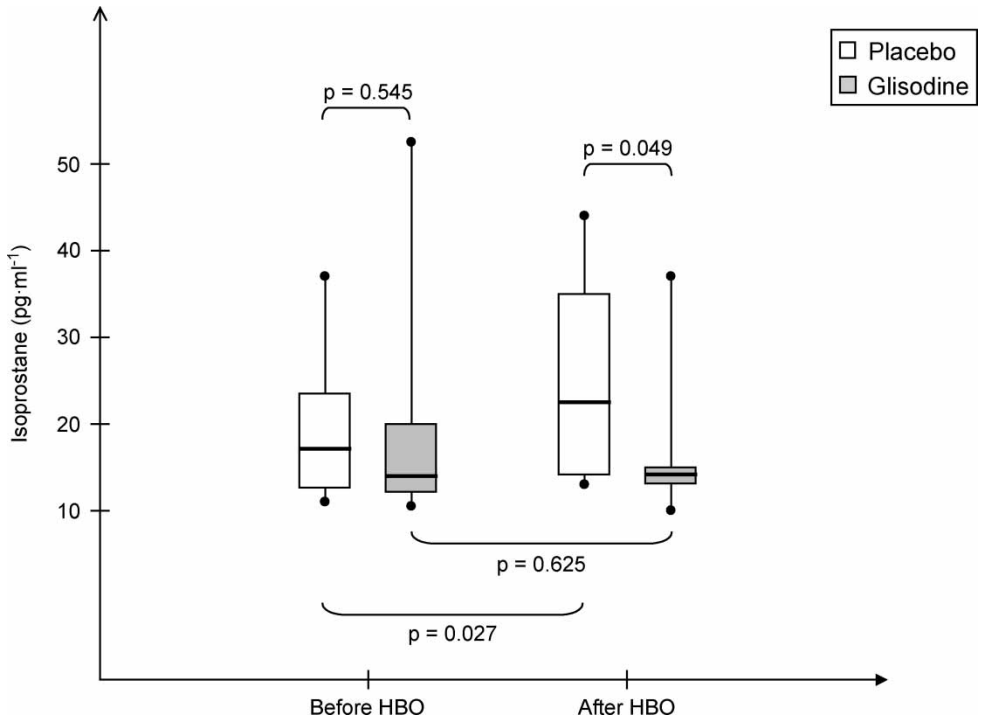

FIGURE 2 *Plasma isoprostrane levels.* Data are presented as boxplots displaying median, quartiles and extremes. Open boxplots represent the data of the placebo-group, grey boxplots represent the data of the Glisodin® group with $n = 10$ in both groups.

TABLE II Parameter of oxidative stress and antioxidant capacity in the blood

		SOD Cu/Zn (U/gHb)	Catalase (kU/gHb)	GPx (U/gHb)	GSH (nmol/ml)	GSSG (nmol/ml)	MDA (nmol/gHb)
Placebo	Before HBO	1328 (1217–1446)	264 (249–301)	64 (58–71)	799 (742–912)	2.0 (1.9–2.5)	4.8 (3.4–8.7)
	After HBO	1398 (1330–1447)	260 (242–283)	65 (59–73)	793 (699–908)	2.5 (1.9–3.3)	3.6 (3.2–5.7)
Glisodine	Before HBO	1343 (1322–1380)	247 (238–275)	56 (50–63)	760 (685–989)	1.7 (1.3–3.2)	4.9 (4.1–7.6)
	After HBO	1356 (1306–1413)	254 (232–286)	55# (50–62)	813 (755–1003)	1.7 (1.2–3.3)	6.1 (4.1–7.4)

All data are median (quartiles), $n = 10$ in both groups, #$p < 0.05$ vs placebo group. SOD, superoxide dismutase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidised glutathione; MDA, malondialdehyde.

the placebo and Glisodin® groups, respectively), HBO significantly increased the plasma isoprostane concentrations in the placebo group (22.3 (14.2–34.9) pg/ml vs baseline). Correspondingly there was a significant increase in the placebo group: from 16.8 (12.4–23.5) pg/ml to 22.3 (14.2–34.9) pg/ml, while no effect was observed in the Glisodin® group: from 14.0 (12.1–19.9) pg/ml to 14.0 (12.9–14.9) pg/ml.

Table II shows that neither SOD and Cat activities nor blood concentrations of GSH and GSSG were significantly affected by the Glisodin® ingestion or by the HBO exposure (Table II). By contrast, blood GPx activity tended to be lower in the Glisodin®-treated subjects already before the HBO exposure ($p = 0.076$), and this difference was significant after the HBO exposure ($p = 0.045$).

DISCUSSION

The present study was undertaken to test the hypothesis whether a new orally effective formulation consisting of wheat gliadin chemically combined with a vegetal preparation of SOD allows preventing DNA strand-breaks affiliated with HBO exposure in healthy volunteers. The SOD–wheat gliadin mixture protected white-blood cell DNA from peroxidation and reduced blood isoprostane levels, while other parameters of oxidative stress in blood remained mostly unaffected.

Exposure to HBO leads to excess formation of oxygen radicals, both *in vitro*[14–16] as well as *in vivo*[2,17] and induces DNA single strand-breaks in cell cultures[18,19] and in blood cells of test subjects.[1,3,10,20] In these studies as well as in the present one, DNA single strand breaks were quantified using the alkaline single cell gel electrophoresis assay ("comet assay"), which allows the detection of DNA single strand-breaks, incomplete excision repair sites and alkali labile sites.[21,22] HBO exposure-related DNA damage in the control group was only moderate in the present study, as compared to previous studies.[1,3,10,20] The fact that all subjects are active scuba divers may have assumed importance in this context. In fact, active scuba divers present an impaired pulmonary diffusion capacity due to cumulative hyperoxia resulting from the repetitive exposures to increased inspired oxygen partial pressures,[23] e.g. enhanced formation of oxygen radicals. Moreover, Rothfuss *et al.*, recently showed that one single HBO exposure triggers adaptive protection against oxidative DNA damage.[24] Consequently, the pre-exisiting diving-associated episodes of hyperoxia in our subjects might have induced a certain degree of protection against HBO-induced DNA single strand breaks, thus resulting in a less pronounced effect of the single HBO-exposure. In addition, such as reviewed previously[25] any other uncontrolled differences related to dietary intake, occupational exposures to reactive oxygen species, smoking and/or variable physical activity may have attenuated the DNA effect of the HBO exposure in the placebo group and/or the intergroup difference. We cannot exclude that the ingestion of a whole foreign protein *per se*, e.g. wheat gliadin, or the antioxidant components of the melon extract[7] assumed importance. Clearly, comparing Glisodin® with a mixture of wheat–gliadine and inactivated SOD would therefore have been preferable. We did not perform such a study because administration of the latter preparation would have required a complete toxicological evaluation prior to its use in human volunteers. Nevertheless it must be underscored that the effect of HBO was close to significance ($p = 0.098$) in the placebo group, while the lack of effect of HBO was clear in the Glisodin®-treated subjects ($p = 0.89$). Moreover, heat inactivation of the SOD activity nearly completely blunted the antioxidant effect of the melon extract in mice fed over 28 days, suggesting that the antioxidant property of the mixture is in fact closely linked to the SOD activity.

Our finding that the SOD–wheat gliadin mixture protected against oxidative stress-induced DNA single strand breaks is underscored by the significantly different post-HBO isoprostane blood levels which had remained stable in the Glisodin®-treated subjects, while they had significantly increased in the placebo group. Isoprostranes are well-accepted markers for oxidative stress,[11] and their formation *in vivo* is regarded to reflect oxidative stress in biological samples more closely than other markers of lipid peroxidation such as MDA.[11,26] This may explain our finding that MDA levels remained

unaffected by the HBO exposure no matter whether placebo- or Glisodin®-treated subjects are concerned. This is further underscored by recent findings reported by Lemaitre *et al.*[27] as well as our own group[3] indicating that normobaric hyperoxia (inspired O_2 fraction 60% over 30 min) or HBO (inspired O_2 fraction 100% at a pressure of 2.5 ATA in a hyperbaric chamber for a total of 3×20 min periods, interspersed with 5 min periods of air breathing) did not affect the venous MDA concentrations. Finally, the significantly lower GPx activity in the Glisodin®-treated subjects also supports our conclusion that the orally effective SOD–wheat gliadin mixture attenuated oxidative DNA stress: higher GPx activity in aorta preparations of mice of various age and dietary regimens was associated with the most pronounced DNA strand breaks.[28]

Not only MDA blood levels remained unchanged in our study, but likewise we did not find any alteration of the whole blood SOD and Cat activities, nor of the GSH and GSSG concentrations. These findings agree with a previous study in human volunteers, which failed to show any effect of a similar HBO exposure on these parameters,[3] while DNA damage as assessed by the comet assay was present.[1,20] It could be argued that subtle alterations of the antioxidant activities and glutathione concentrations might have been missed because measurements were performed in whole blood samples rather than in leukocytes so that a putative effect was masked by the high background enzyme concentrations in plasma and in erythrocytes.[29] We did not assess antioxidant enzyme activities or glutathione content in isolated leukocyte preparations because of the high amount of blood required for this purpose. In contrast to animals[6] we did not find significant changes in blood SOD and Cat activities after oral SOD ingestion. Assuming a total blood volume of about 8% of body weight and a hemoglobin content of about 160 g/l in healthy male subjects the measured red cell SOD activity of approximately 1300 U/gHb (pre HBO value in the placebo group, Table II) would yield an erythrocyte SOD activity of about 1.5×10^6 U. The administered dose of SOD in our study was 1000 UI-NBT per day, e.g. less than 1‰ of the total red cell SOD activity. Consequently, it was unlikely that the administered dose would significantly change this value. In fact, in animal experiments increased SOD activity was detectable both in erythrocytes and plasma, when Glisodin® was administered in a higher dose and over a longer period of time.[5,6] Nevertheless, the present data indicate a clear-cut biological effect leading to conclude that this orally effective mixture of vegetal SOD and wheat gliadin prevents leukocyte DNA damage following HBO. This finding raises the question of the putative mechanism of action of Glisodin® when administered by oral route. Indeed, while vegetal SOD, as any orally administered protein, is usually deteriorated by gastric digestion and thus ineffective,[4] combining SOD with wheat gliadin renders it biologically effective. In addition, the vehicle gliadin *per se* might also have an additional effect at the gut wall level. It has been recently shown that gliadin was able to activate zonulin release by intestinal epithelial cells[30] that subsequently generate a PKC-mediated cytoskeleton reorganization. This finding is of importance since zonulin was reported to be responsible for tight-junctions opening and thus increase in intestinal permeability.[31] Hence, gliadin may possibly favour the vegetal SOD to cross the gut barrier. The question remains, how heterologous SOD can protect leukocytes from oxidative stress related DNA strand breaks. One hypothesis could involve the hydrogen peroxide formation resulting from SOD activity of Glisodin®, which may trigger a pro-oxidant signal leading to reinforce the endogenous anti-oxidant defence. Although the lack of effect in the present work of a two-week treatment with Glisodin® on circulating antioxidant enzymes does not support this latter suggestion, the dosage and the duration of Glisodin® administration as well as the absence of blood leukocyte purification do not permit to rule out this proposal. Therefore, further studies are needed to clarify the mechanism of action of this new formulation of a mixture of heterologous vegetal SOD and wheat gliadin, which is effective even when administered orally.

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