Formation of Non–cyclooxygenase-derived Prostanoids (F2-Isoprostanes) in Plasma and Low Density Lipoprotein Exposed to Oxidative Stress In Vitro

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Abstract

F2-isoprostanes are prostaglandin F2-like compounds that are known to be formed in vivo by free radical oxidation of arachidonyl-containing lipids, and their plasma levels have been suggested as indicators of in vivo oxidative stress. As oxidation of LDL, a likely causal factor in atherosclerosis, involves lipid peroxidation, we investigated whether F2-isoprostanes are formed in plasma and LDL exposed to oxidative stress, and how F2-isoprostane formation is related to endogenous antioxidant status. In plasma exposed to aqueous peroxyl radicals, lipid hydroperoxides and esterified F2-isoprostanes were formed simultaneously after endogenous ascorbate and ubiquinol-10 had been exhausted, despite the continued presence of urate, α-tocopherol, β-carotene, and lycopene. In isolated LDL exposed to aqueous peroxyl radicals or Cu2⁺, consumption of endogenous ubiquinol-10 and α-tocopherol was followed by rapid formation and subsequent breakdown of lipid hydroperoxides and esterified F2-isoprostanes, and a continuous increase in LDL’s electronegativity, indicative of atherogenic modification. In Cu2⁺-exposed LDL, the decrease in esterified F2-isoprostane levels was paralleled by the appearance of free F2-isoprostanes, suggesting that hydrolysis by an LDL-associated activity had occurred. Our data suggest that F2-isoprostanes are useful markers of LDL oxidation in vivo. As F2-isoprostanes are potent vasoconstrictors and can modulate platelet aggregation, their formation in LDL demonstrated here may also have important implications for the etiology of cardiovascular disease. (J. Clin. Invest. 1994. 93:998–1004.) Key words: atherosclerosis • free radicals • lipid peroxidation • antioxidants • eicosanoids

Introduction

Oxidation of LDL has been suggested to play a causal role in atherosclerosis (1, 2). Evidence in support of this hypothesis includes the immunochromatographic detection of oxidized LDL in atherosclerotic lesions (3, 4), extraction of oxidized LDL from such lesions (3, 5), and the presence in human plasma of circulating autoantibodies specific for epitopes on oxidized but not native LDL (3, 6). In vitro studies have shown that oxidatively modified LDL, in contrast to native LDL, is accumulated rapidly by monocyte-macrophages to generate the lipid-laden foam cells (7–10) characteristic of fatty streak lesions observed in early atherosclerosis (11). Furthermore, in addition to direct involvement in foam cell genesis, oxidized LDL exhibits a wide range of atherogenic properties including stimulation of leukocyte adherence to the microvascular endothelium (12), induction of monocyte chemotactic protein-1 in endothelial and smooth muscle cells (13), inhibition of endothelium-dependent vasodilation (14, 15), and regulation of vascular hemodynamic activity (16, 17).

One of the main characteristics of LDL oxidation is lipid peroxidation (18), and autooxidation of fatty acids in vitro has been shown to generate prostaglandin-like bicyclic endoperoxides (19, 20). More recently (21) it has been demonstrated that, under certain ex vivo conditions, plasma arachidonyl-containing lipids may form a series of prostaglandin F2-like compounds (F2-isoprostanes). F2-isoprostane formation from arachidonic acid has also been shown to occur in vivo in humans by a free radical-mediated process that is cyclooxygenase independent (22). Furthermore, administration of carbon tetachloride or diquat to experimental animals, hence exposing them to oxidative stress, results in marked elevation of plasma levels of F2-isoprostanes (23). F2-isoprostanes are initially formed in situ esterified to phospholipids and subsequently released preformed (24).

In contrast to lipid hydroperoxides, which readily decompose and cannot be detected in human plasma with a sensitive chemiluminescence assay (25), F2-isoprostanes are chemically stable end-products of lipid peroxidation (21) and are present at significant steady-state concentrations both in human plasma and urine (22). Thus, measurement of plasma F2-isoprostane levels may provide a sensitive, specific, and noninvasive method for assessment of in vivo lipid oxidative damage in humans (26). In addition to serving as markers of endogenous lipid peroxidation, there can be biological consequences associated with the formation of F2-isoprostanes. One of the F2-isoprostanes that has been tested, 8-epi-prostaglandin F2α, has been found to be a potent vasoconstrictor and to modulate platelet aggregation (22, 27–29). Therefore, F2-isoprostanes may also influence certain pathophysiological processes involved in atherogenesis.

The aim of the studies described herein was to characterize the formation of lipid esterified and free F2-isoprostanes in human plasma and isolated LDL exposed to both metal ion–dependent and –independent forms of oxidative stress and to examine the relationship of F2-isoprostane formation in these experimental systems to antioxidant status, formation of lipid hydroperoxides, and oxidative modification of LDL.

Methods

Materials. Sodium heparin Vacutainers were purchased from Becton Dickinson (Rutherford, NJ), Acrodisc LC13 syringe filters from Gel-
man Sciences Inc. (Ann Arbor, MI), Sephadex G-25M PD-10 columns from Pharmacia (Uppsala, Sweden), LC-18 and LC-Si solid phase extraction (SPE) columns and microreaction vials from Supelco Inc. (Bellefonte, PA), and Lipo gels for agrose gel electrophoresis of LDL from Beckman Instruments Inc. (Brea, CA). Chelex-100 resin was purchased from Bio-Rad Laboratories (Richmond, CA), 2.2'-azobis(2-aminopropane) hydrochloride (AAPH) from Kodak (Rochester, NY), and 15-hydroperoxy-ecosatetraen-l-oic acid from Cayman Chemical Co. Inc. (Ann Arbor, MI). Organic solvents were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

**LDL isolation.** After an overnight fast, blood from a healthy normolipidemic male volunteer was collected in a Vacutainer tube (286 USP U sodium heparin/15 ml blood) and centrifuged (500 g for 20 min at 7°C) to give plasma. LDL was isolated from fresh plasma by single vertical spin discontinuous density ultracentrifugation (30). Plasma density was adjusted to 1.21 g/ml with solid KBr (0.3265 g/ml plasma), and a discontinuous NaCl/KBr gradient was established in centrifuge tubes by layering the density-adjusted plasma (1.5 ml) under 0.154 M NaCl (3.5 ml). The samples were centrifuged at 80,000 rpm for 45 min at 7°C in a rotor (Near Vertical Tube 90; Beckman Instruments Inc.) (443,000 g, L-80M ultracentrifuge; Beckman Instruments Inc.) using slow acceleration and deceleration modes (14). After centrifugation, LDL was observed as an orange band in the upper middle portion of tubes and was removed by puncturing tubes with a hypodermic needle and withdrawing the LDL into a syringe. Contaminating metal ions were removed from the isolated LDL by addition of a small quantity of Chelex-100 resin with gentle mixing. The resin was removed by centrifugation (500 g for 1 min at 7°C) and the supernatant, containing LDL, was filtered (0.2 μm Acrodisc filter; Gelman Sciences Inc.). KBr and potential low molecular weight contaminants (e.g., ascrobate) were removed from LDL preparations by sequential passage through two or three Sephadex G-25M PD-10 gel filtration columns according to the manufacturer's instructions. LDL protein was determined by a modification (31) of the Lowry method (32).

**Experiments.** Fresh plasma was incubated at 37°C under air for 0–5 h with 50 mM AAPH, a water-soluble azo compound that thermally decomposes to generate peroxyl radicals at a constant rate (33). Aliquots were removed at various time intervals and, after termination of the reaction by transfer to an ice bath, were analyzed for water-soluble antioxidants, cholesterol ester hydroperoxides (CEOOH), phospholipid hydroperoxides (PLOOH), and F2-isoprostanes. Control incubations were identical to experimental incubations but contained an equal volume of the vehicle 10 mM PBS, pH 7.4, instead of AAPH.

Isolated LDL was exposed to either a metal ion–independent (AAPH) or metal ion–dependent (Cu2+) oxidative stress. In these experiments, LDL (protein = 0.2 mg/ml) was incubated at 37°C under air for 0–20 h with either 10 nM AAPH or 10 μM CuCl2 and 2.5 μM of the metal chelator diethylenetriaminepenta-acetic acid (DTPA). The latter was added to prevent trace amounts of contaminating metal ions from contributing to oxidation of LDL in incubations with AAPH, and in control incubations containing no AAPH or no added Cu2+. Aliquots were removed from the incubations at various time intervals, and after termination of the reaction by transfer to an ice bath, (AAPH) or addition of DTPA to a final concentration of 1.0 mM (Cu2+), they were analyzed for lipid-soluble antioxidants, CEOOH and PLOOH, F2-isoprostanes, and electrophoretic mobility on agarose gels. Control incubations were identical to experimental incubations but contained the vehicle PBS instead of AAPH or Cu2+.

**Measurement of F2-isoprostanes.** Free F2-isoprostanes in plasma or LDL were quantitated after purification and derivatization by selected ion monitoring gas chromatography negative ion chemical ionization/mass spectrometry employing [1H2]PGF2α as an internal standard (21). Compounds were analyzed by pentafluorobenzyl ester, trimethylsilyl ether derivatives monitoring the M-181 ions, m/z 569 for endogenous F2-isoprostanes and m/z 573 for [1H2]PGF2α. The F2-isoprostanes elute as a series of chromatographic peaks over a 20-s interval and quantitation is based on the primary peak eluting 7 s before the internal standard. To determine the concentrations of esterified F2-isoprostanes, plasma or LDL were extracted by a modified Folch procedure and base hydrolyzed (21) before quantitation of the resulting free F2-isoprostanes as described above.

**Measurement of lipid hydroperoxides and antioxidants.** CEOOH and PLOOH in plasma and LDL were quantitated using an HPLC method with chemiluminescence detection (25, 34) and 15-hydroperoxy-ecosatetraen-l-oic acid as a standard. Water-soluble and lipid-soluble antioxidant concentrations were determined by HPLC with electrochemical detection (35, 36).

**Electrophoretic mobility of LDL.** Increased anodic electrophoretic mobility of LDL on agarose gels was used to assess oxidative modification (18). LDL was electrophoresed at 100 V for 0.5 h in 0.5% agarose gels (Lipo gels; Beckman Instruments Inc.) with 0.05 M barbital buffer, pH 8.6, in an electrophoresis system (Paragon; Beckman Instruments Inc.). Gels were fixed, stained with Sudan black B and destained according to the manufacturer's instructions.

**Results.**

Incubation of plasma at 37°C with AAPH, a source of aqueous peroxyl radicals (33), resulted in a rapid decline of endogenous ascorbate and ubiquinol-10 to undetectable levels within 1 h (Fig. 1 A). In contrast, plasma α-tocopherol and urate concentrations were essentially unchanged for up to 3 h of incubation, and only modest decreases in lycopene and β-carotene levels were observed (Fig. 1 A). With the exceptions of ascorbate and ubiquinol-10, the concentrations of which decreased to 69% and 54% of their initial values, respectively, plasma antioxidant levels remained essentially constant for 3 h in control incubations without AAPH (data not shown).

Both CEOOH and PLOOH were not detectable ( <2 nM), and esterified F2-isoprostanes remained at a low baseline level ( ~0.10 nM) in plasma incubated with AAPH for up to 1 h (Fig. 1 B). After this time, when ascorbate and ubiquinol-10 were no longer detected, the levels of lipid hydroperoxides and esterified F2-isoprostanes began to increase. The rates of lipid peroxidation and esterified F2-isoprostane formation were initially low (t = 1–2 h), but subsequently increased. The concentrations of esterified F2-isoprostanes formed were about four orders of magnitude lower than those of lipid hydroperoxides. Lipid hydroperoxides were undetectable for up to 5 h in control incubations without AAPH. Similarly, plasma levels of esterified F2-isoprostanes remained constant in control incubations for up to 5 h (0.09 nM compared with 0.10 nM at t = 0 h). Free F2-isoprostane levels were also measured and were found to be virtually unaffected by incubation of plasma with AAPH for 3 h (0.08 nM compared with 0.02 nM at t = 0 h). This result is not unexpected as free fatty acids, potential precursors of free F2-isoprostanes, are present only in small concentrations in plasma and are bound to albumin, where they are protected against oxidative damage (37).

Incubation of isolated LDL with either AAPH or Cu2+ caused endogenous ubiquinol-10 levels to decrease precipitously so that within 0.5 h this compound was no longer detectable (Figs. 2 A and 3 A). Consumption of ubiquinol-10 was followed by consumption of α-tocopherol, and then lycopene and β-carotene (Figs. 2 A and 3 A). While ubiquinol-10 levels...
decreased in control incubations to 43% of the initial concentration, all other antioxidant levels remained essentially unchanged for 3 h (data not shown).

Levels of lipid hydroperoxides and esterified F2-isoprostanes in LDL remained low during the first 0.5 and 1 h, respectively, of incubation with AAPH or Cu2+ (Figs. 2 B and 3 B), coinciding with the consumption of ubiquinol-10 and α-tocopherol (Figs. 2 A and 3 A). Thereafter, the rates of CEEOH, PLOOH, and esterified F2-isoprostane formation increased rapidly. In LDL exposed to AAPH, CEEOH, and esterified F2-isoprostane levels reached a plateau after 1.5–2 h before starting to decrease after 4 h of incubation, while PLOOH levels remained virtually unchanged for up to 7 h of incubation (Fig. 2 B). In contrast, in LDL exposed to Cu2+, the concentrations of CEEOH and esterified F2-isoprostanes decreased sooner and more rapidly, and PLOOH levels also decreased, albeit only slowly (Fig. 3 B). Similar to plasma, the levels of esterified F2-isoprostanes formed in LDL were more than three orders of magnitude lower than those of lipid hydroperoxides. In control incubations of LDL without AAPH or Cu2+, lipid hydroperoxides remained undetectable (i.e., <10 pmol/mg protein), while esterified F2-isoprostane levels were extremely low (0.15 pmol/mg protein) and remained essentially unchanged for up to 7 h of incubation.

The time-dependent changes in relative electrophoretic mobility (REM) of LDL, an indicator of its atherogenic modification (1, 18), are shown in Figs. 4 and 5 for incubations containing AAPH or Cu2+, respectively. For LDL incubated with AAPH (Fig. 4), no increase in REM was observed until 1 h after initiation of the incubation, the time at which significantly increased levels of lipid hydroperoxides and esterified F2-isoprostanes were first observed (Fig. 2 B). Thereafter, REM increased continuously despite an observed decrease in...
lipid hydroperoxides and esterified \( F_2 \)-isoprostane levels between 4–20 h of incubation. A similar trend was observed for the REM of LDL incubated with Cu\(^{2+} \) (Fig. 5): before formation of significant quantities of lipid hydroperoxides and esterified \( F_2 \)-isoprostanes (\( t = 0–1 \) h) there was no increase in REM; this initial lag phase was followed by a continuous increase in REM for up to 20 h, a period during which rapid formation and decomposition of lipid hydroperoxides and esterified \( F_2 \)-isoprostanes was observed. LDL from control incubations without AAPH or Cu\(^{2+} \) showed no increase in REM for up to 20 h (Fig. 5).

Finally, we investigated whether the decrease in esterified \( F_2 \)-isoprostane levels in LDL exposed to oxidative stress (Figs. 2 B and 3 B) was caused by hydrolysis. To this end, LDL was incubated with Cu\(^{2+} \), and the concentrations of both esterified and free \( F_2 \)-isoprostanes were followed during 20 h (Fig. 6). Concomitant with the decrease in esterified \( F_2 \)-isoprostane levels between 1 and 20 h of incubation, there was a continuous increase in the levels of free \( F_2 \)-isoprostanes, suggesting that hydrolysis did occur.

**Discussion**

In this study, we investigated antioxidant consumption and formation of lipid hydroperoxides and \( F_2 \)-isoprostanes in human plasma and LDL incubated under two different types of oxidizing conditions. In agreement with our earlier studies (35, 37), we found that in plasma exposed to a constant flux of aqueous peroxyl radicals no detectable amounts of lipid hydroperoxides were formed until ascorbate was depleted (Fig. 1). Ubiquinol-10, a lipid-soluble antioxidant present in small concentrations in plasma (25, 38), was consumed together with ascorbate. Similar to lipid hydroperoxides, concentrations of esterified \( F_2 \)-isoprostanes only increased after complete consumption of ascorbate and ubiquinol-10. Lipid peroxidation and esterified \( F_2 \)-isoprostane formation occurred despite the continued availability of other antioxidants in plasma at physiological concentrations; i.e., urate, \( \alpha \)-tocopherol, lycopene, and \( \beta \)-carotene. It is particularly noteworthy that in our experiments, \( \alpha \)-tocopherol concentrations remained virtually unchanged even after ascorbate had been exhausted, suggesting that the antioxidant effect of ascorbate in plasma is not simply caused by regeneration of \( \alpha \)-tocopherol. A synergistic antioxidant interaction between ascorbate and \( \alpha \)-tocopherol has been observed in incubations of phospholipid liposomes (39) and
Figure 6. Cu

2+

-dependent formation of free and esterified F2-isoprostanes in LDL. LDL (protein = 0.2 mg/ml) was incubated with 10 μM Cu

2+

and 2.5 μM DTPA at 37°C for 0–20 h and aliquots were removed at intervals for quantitation of esterified (●) and free (○) F2-isoprostanes which are reported in nM concentrations. In a second experiment performed under identical conditions, esterified F2-isoprostane levels increased from 0.2 nM to 12.8 nM during incubation from 0 to 3.5 h, respectively, before decreasing to 5.1 nM after 20 h of incubation, while free F2-isoprostane levels increased continuously from 0.01 nM (t = 0 h) to 4.0 nM (t = 20 h).

LDL (40), and has been suggested to also occur in plasma (41, 42). In contrast, our data indicate that in plasma ascorbate functions independently of α-tocopherol by directly scavenging aqueous peroxyl radicals before they can attack the lipoprotein lipids (35, 43), thus effectively preventing lipid peroxidation and formation of esterified F2-isoprostanes. In addition, ascorbate may prevent initiation of lipid peroxidation in LDL by a second mechanism; i.e., stable modification of the lipoprotein by ascorbate oxidation product(s) (44).

In isolated LDL, where ascorbate is absent, we found that ubiquinol-10 formed the first line of antioxidant defense, both under metal ion-independent (AAPH) and metal ion–dependent (Cu

2+

) oxidizing conditions. We have shown previously that in LDL incubated with AAPH, ubiquinol-10 is consumed before the other LDL-associated antioxidants, and that the rate of lipid peroxidation is low as long as ubiquinol-10 is present (36). Since ubiquinol-10 is only a minor antioxidant in LDL, and is present at much lower concentrations than α-tocopherol (45), the antioxidant protection afforded by ubiquinol-10 may be relevant to the prevention of formation of minimally modified LDL (13, 16, 46), but not of the more extensively oxidized LDL recognized by macrophage scavenger receptors and contributing to foam cell formation (1, 2). Minimally modified LDL has been shown to upregulate monocyte chemotactic protein-1 and colony-stimulating and tissue factors in human endothelial and smooth muscle cells, and, thus, may play a critical role in the beginning stages of atherosclerosis (13, 16, 46).

In contrast to ubiquinol-10, α-tocopherol plays a significant role in protecting LDL against Cu

2+

-induced extensive oxidative modification (45). α-Tocopherol is by far the most abundant antioxidant in LDL (18, 45) and, hence, the absolute amounts consumed during the initial stages of LDL oxidation are substantially greater than the amounts of ubiquinol-10 consumed (see Figs. 2 A and 3 A, and accompanying legends). Interestingly, when LDL is exposed to a low flux of aqueous peroxyl radicals, α-tocopherol seems to act as a prooxidant, rather than an antioxidant (45, 47). In the present study, high concentrations of AAPH were used, and α-tocopherol protected against, rather than promoted, LDL oxidation (Fig. 2). This observation is in agreement with the findings of Bowry et al. (47), who have shown that with increasing AAPH concentrations α-tocopherol’s effect in LDL switches from pro- to antioxidant. However, in contrast to the situation in isolated LDL, in plasma incubated with a high concentration of AAPH, α-tocopherol was ineffective against lipid peroxidation and formation of esterified F2-isoprostanes (Fig. 1). It may be that in plasma the AAPH-derived peroxyl radicals react primarily with aqueous target molecules such as hydrophilic antioxidants and thiol groups on albumin (37) and that, therefore, the actual rate of radical attack on the lipoproteins is very low, resulting in a lack of antioxidant effect by α-tocopherol.

Once ubiquinol-10 and α-tocopherol in isolated LDL had been exhausted, there was rapid formation of lipid hydroperoxides and esterified F2-isoprostanes. β-Carotene and lycopene, present in LDL in small concentrations similar to those of ubiquinol-10, appeared to be of little importance in preventing LDL oxidation, in agreement with our previous data (45). The formation of esterified F2-isoprostanes closely paralleled that of lipid hydroperoxides, consistent with the concept that arachidonyl hydroperoxides are the precursors of esterified F2-isoprostanes (21, 22). Only a small fraction (i.e., 0.05–0.1% [500–1,000 ppm]) of the lipid hydroperoxides formed in LDL were converted to esterified F2-isoprostanes, and a similarly low conversion rate was observed for plasma (≈0.02%, or 200 ppm). It is interesting to note that formation of F2-isoprostanes involves reduction of lipid endoperoxides to alcohol groups (21, 22), and that this reduction apparently can occur in plasma and LDL concomitant with oxidation of lipids (i.e., in an oxidizing environment), and in the absence of measurable antioxidant levels in LDL. Further studies are needed to identify the biological reducing agent(s) and to elucidate the reaction mechanism.

Formation of lipid hydroperoxides and esterified F2-isoprostanes in LDL was followed by their decomposition, which was particularly fast in incubations of LDL with Cu

2+

. Lipid hydroperoxides are known to decompose readily, a process catalyzed by transition metal ions, to form a variety of toxic aldehydeic species (18). These aldehydes, such as malondialdehyde and 4-hydroxynonenal, can form Schiff bases with lysine residues of apolipoprotein B, resulting in increased anodic electrophoretic mobility of LDL and increased uptake by macrophages via the scavenger receptor pathway (1). Consistent with such a mechanism of oxidative modification of LDL, in our experiments, there was a continued increase in LDL’s electronegativity during the decomposition of lipid hydroperoxides. In contrast to the disappearance of lipid hydroperoxides, the observed decrease in levels of esterified F2-isoprostanes cannot be explained on the basis of chemical decomposition, as these compounds are very stable (21). Our finding that free F2-isoprostane levels in Cu

2+

-exposed LDL increased concomitantly with the disappearance of esterified F2-isoprostanes (Fig. 6) indicates that esterified F2-isoprostanes formed in LDL are subsequently hydrolyzed, conceivably by a phospholipase A2 activity intrinsic in apolipoprotein B (48) or by platelet-activating factor acetylhydrolase activity associated with LDL (49). Similarly, in vivo formation of F2-isoprostanes occurs in situ on tissue phospholipids, from which they are released in free form, presumably by a phospholipase(s) (24).

In summary, we have found that in plasma exposed to aqueous peroxyl radicals, complete consumption of the endoge-
nous antioxidants ascorbate and ubiquinol-10 is immediately followed by simultaneous formation of lipid hydroperoxides and esterified F2-isoprostanes. In isolated LDL exposed to either metal ion-dependent or -independent oxidizing conditions, lipid hydroperoxides and esterified F2-isoprostanes are formed at substantially increased rates after depletion of LDL-associated ubiquinol-10 and α-tocopherol, and free F2-isoprostanes appear to be formed subsequently from their esterified precursors by hydrolysis. As shown previously, levels of esterified F2-isoprostanes in various organs and levels of both free and esterified F2-isoprostanes in plasma of animals increase dramatically in association with oxidative stress in vivo and correlate with the extent of tissue injury (23). For example, plasma levels of esterified F2-isoprostanes in rats treated with carbon tetrachloride increase up to 50-fold compared to baseline (23, 24). In addition, as shown here, F2-isoprostanes esterified to lipids can be detected in human plasma and LDL derived from it. Taken together, these data suggest that plasma levels of F2-isoprostanes may be used as indicators of lipid peroxidation and oxidative modification of LDL in vivo (26), and, thus, possibly of an individual’s risk of atherosclerotic disease (1, 6). The formation of F2-isoprostanes in LDL, and its inhibition by ascorbate, ubiquinol-10, and α-tocopherol, may itself contribute significantly to the progression of cardiovascular disease or prevention thereof, respectively, as at least one of these F2-isoprostanes, 8-epi-prostaglandin F2α, exerts potent vasconstrictor activity in vivo (22, 27) and modulates platelet activation (27–29) at concentrations similar to or lower than those observed in the present study.

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