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Antioxidant Effects of Whey Protein as a Dietary Supplement To Alleviate Cadmium-Induced Oxidative Stress in Male Wistar Rats

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Abstract

The liquid whey is a byproduct produced during cheese making. Cadmium is a highly hazardous heavy metal with cumulative toxic effects. The present research work was done to clarify the possible role of whey proteins in alleviating cadmium-induced oxidative stress. The used rats were allotted equally and randomly into three experimental groups; untreated control, cadmium-exposed, and cadmium-exposed and whey protein-administered groups. The biochemical and haematological assays of rats exposed to cadmium (group 2) manifested significant alterations compared to those of untreated control animals. Concerning the biochemical serum profile, group 3 animals showed relatively increased levels of total proteins, significant increments of total thiols, glutathione, total antioxidant capacity (TAC), and catalase, and significant decrements in the levels of blood cadmium, alanine transferase (ALT), aspartate transferase (AST), alkaline phosphatase (ALP), creatinine, urea, bilirubin, hydrogen peroxide (H_2O_2), and malondialdehyde (MDA) compared to the animals exposed to cadmium (group 2). Homogenates of liver and kidney tissues obtained from group 3 animals demonstrated similar results to that revealed by the serum assay. It was concluded that whey proteins as a dietary supplement can offer potential antioxidant properties that enable these supplementary proteins to alleviate cadmium-induced oxidative stress.

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Introduction

Cadmium is a highly hazardous heavy metal that constitutes a source of environmental pollution. It is used in a considerable number of diverse industries. Cadmium-induced chronic toxicity is featured by progressive drastic effects due to a gradual accumulation of the toxic heavy metal in different tissues.[1] Cadmium toxicity can provoke oxidative stress with the associated damaging effects in the involved tissues and organs, especially kidneys and liver.[2]

The liquid whey is raised as a byproduct from cheese making and can be exposed to sequential filtration steps to prepare special whey products. The Initial filtration and purification are performed to remove the majority of fat and lactose contents, and the final ultrafiltration is done to increase the protein concentration to be higher than 90% in some whey products.

Oxidative activities are among the main events that eventually lead to cell and tissue damage. Inhibition and/or prevention of these activities is an ultimate function of the antioxidative mechanisms. Humans and animals possess a naturally efficient antioxidant system for detection of the oxidative metabolites, such as free radicals, and preventing their damaging effects.[2-6] In other words, under physiological conditions there is a continuous equilibrium between the oxidation reactions and antioxidation bioactivities to avoid possible cellular damage and to maintain the integrity and functions of cells and tissues.

Natural antioxidants are greatly concerned,[7] and their medical applications have been used for the prevention of some major disease conditions including cancer, cardiovascular disorders, Alzheimer's, and arthritis and also its role in the alleviation of ageing manifestations.[8-13]

Proteins that can protect the cells and tissues from oxidative damaging effects are known as antioxidant proteins.[14]These antioxidant proteins are not synthesized in the body and external sources of such type of proteins, as well as other micronutrients, are essential to sustain the status of oxidation-antioxidation balance.[15-17]

Previous *in vivo* and *in vitro* studies related to the antioxidant properties of whey proteins [5,14,18-25] targeted the capability of these external sources of proteins to enhance the cellular antioxidative activities, but were not focused on the role of whey proteins in case of toxicity. Therefore, there is a need for a research trial to clarify some aspects of the possible roles of whey proteins as external rich protein sources to enhance and maintain the endogenous antioxidative mechanisms in cases of heavy metal toxicity.

The present study aims to investigate the possible roles of whey protein in alleviating the oxidative stress induced by a highly toxic heavy metal.

Materials and Methods

Experimental Animals

Adult Wistar male rats (n=60), aged four months and weighed between 170-210 g, were used in the present experimental work. The standard laboratory environment was provided to maintain the used animals (temperature 24 ± 1 °C, 12 hr dark/light cycle, and 35-70% humidity). The guidelines that were officially established by the Ethics Committee (Imam Mohammad ibn Saud University, Saudi Arabia) to maintain lab animals were strictly followed (Lab-Animals-022-198).

Cadmium

Analytical grade of cadmium chloride (Cd Cl2) (Merck, Germany) was used. Cadmium was dissolved in purified water to prepare an aqueous solution of a final concentration of 5 mg / mL.

Whey Protein

Whey protein (WP-CB001) (certified reference material) was procured from Sigma-Aldrich (Darmstadt, Germany). This product is considered an impact whey isolate, and certified by the Federal Institute of Metrology METAS, Switzerland (Swiss National Metrology Institute). Whey protein was dissolved in purified water to prepare an aqueous solution of a final concentration of 50 mg / mL.

Experimental Design

The rats were acclimatized for one week and allotted randomly into three equal groups (n=20 / group), designated Groups 1, 2 and 3. Group 1 served as untreated control, the control animals were neither exposed to cadmium nor received whey protein. An aqueous solution of cadmium chloride (5 mg/kg body weight/day) was administered orally to Group

2 animals (oral gavage, 1 mL/kg body weight). The same dose of cadmium was administered orally to Group 3 animals along with whey protein at the total oral dose of 200 mg/day using an oral gavage (1 mL/kg body weight). A gap time of 6 hr was adjusted between administration of cadmium and whey protein.

The experiment period was 6 weeks, and throughout dry ration and water were available *ad libitum*.

Behavioural activity, feed consumption, water intake of all experimental animals, and any manifested clinical signs were closely monitored.

Hematological and Biochemical Assays

Animals were anaesthetized (3% isoflurane) at the end of the experiment, and cardiac puncture was used to collect the blood from all animals. The various haematological indices were estimated in the anticoagulated blood samples (EDTA). Serum, separated from the coagulated blood samples, was removed and stored at -20 ^{o}C until the time of biochemical assay. After collection of the required blood samples, animals were killed by decapitation, and liver and kidney tissues were removed and homogenized in 150 mM NaCl. The tissue homogenates were centrifuged at 3000 Xg at 4^{o}C for 10 min, and the collected supernatants were used to assess the various biochemical parameters that reflect the oxidative stress and its alleviation in the hepatic and renal tissues.

Blood Cadmium Level

Blood samples (1 mL) were digested by using a mixture of (perchloric acid) HClO4- and nitric acid (HNO_3), to determine blood cadmium levels by employing an atomic absorption spectrophotometer (CBC 906 AA) as described in a previous study.[26]

Hematological Assay

Red blood cell (RBCs) and total white blood cell (WBCs) counts, haemoglobin (Hb) concentration, and packed cell volume (PCV) percentage were measured using the anticoagulated blood samples. The total leukocyte and erythrocyte counts were measured using a hemocytometer. PCV percentage was measured using the micro-hematocrit method and Hb concentration was estimated by the Cyanmet-hemoglobin method as previously described.[27,28]

Biochemical Assay

The assayed biochemical parameters were represented by the serum levels of total proteins, albumin, globulin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, urea, blood urea nitrogen (BUN), total thiols, catalase, glutathione (GSH), total antioxidant capacity (TAC), malondialdehyde (MDA), and hydrogen peroxide (H_2O_2).

GSH was estimated using a glutathione colourimetric assay kit (ElabScience, USA). Glutathione assay is based on the reduction of GSSG to GSH by glutathione reductase, and subsequent reaction of GSH with a kit component to produce a yellow product. The OD value of this reaction product is proportional to the level of glutathione in the tested sample.

A total thiol colourimetric assay kit (Cell Biolabs Inc., USA) was used to estimate total thiols. Catalase level was determined using a catalase colourimetric assay kit (BioVision; Abcam, UK).

TAC was determined using a TAC assay kit (Sigma-Aldrich, Germany). The principle of this analytical kit is to determine the combined protein and small-molecule antioxidants or the concentration of only small molecules of antioxidants. Small molecules and proteins convert Cu^{2+} to Cu^{+}. The inserted protein mask as a component of the kit prevents the action of proteins on Cu^{2+}, and only small-molecule antioxidants act on Cu^{2+}. A colourimetric probe chelates the reduced Cu^{+} ions, and the resultant absorbance peak represents these reduced ions and comes in proportion to the antioxidant capacity. MDA level was measured by a colourimetric assay kit for MDA (Elabscience, USA). The detection method is based on the reaction of MDA (in the catabolite of lipid peroxide) with thiobarbituric acid (TBA), a kit component, producing a red compound. The absorbance peak of this compound is proportional to the level of MDA in the tested sample.

Hydrogen peroxide (H_2O_2) level was determined using H_2O_2 colourimetric assay kit (Elabscience, USA).

The levels of ALT, AST, and ALP were estimated by the relevant diagnostic kits (BioMeerieux,

France). BUN level was assessed by a colourimetric detection kit (ThermoFisher Scientific, USA). The urea level was measured by a colourimetric assay kit (BioVision, Biovision Incorporated, UK). The remaining biochemical parameters, including total proteins, creatinine, bilirubin, albumin and globulin were estimated using the corresponding colourimetric diagnostic kits (Interchim Diagnostics Biochemistry kits, France).

Homogenates of hepatic and renal tissues were analyzed for the levels of total thiols, glutathione, catalase, H_2O_2, MDA and TAC in the tissues. The assay kits employed to determine these parameters in the serum were used to determine their levels in the tissue homogenates.

Statistical Analysis

All data extracted from the performed assays are presented as means ± S.D. The data from all animals were analyzed using ANOVA by applying statistical analysis SPSS software (SPSS Inc. Chicago IL, USA) for data comparisons of means between multiple groups. The Dunn-Bonferroni test was used as a post-hoc test. Differences with a P-value less than 0.05 ($P < 0.05$) were considered statistically significant.

Results

The mean blood cadmium level was (0.0021 ± 0.0001 ppm) in control untreated rats, measured (0.493 ± 0.023 ppm) (significant increase, $P < 0.05$) in rats intoxicated with cadmium and estimated (0.204 ± 0.026 ppm) (significant decrease, $P < 0.05$) in rats exposed to cadmium and given whey protein.

Rats treated with cadmium and did not receive whey protein (group 2) had decreased haematological indices; the most noticeable decrements were that of haemoglobin concentration and percentage of packed cell volume. These parameters were significantly increased in rats exposed to cadmium and administered with whey protein (group 3) concerning cadmium-exposed animals (group 2).

Table (1) shows the haematological changes in rats exposed to cadmium, and rats exposed to cadmium and administered with whey protein compared to the untreated control rats.

Table 1:Hematological changes in rats exposed to cadmium, and rats exposed to cadmium and administered with whey protein compared to the untreated control rats (n= 20 animals/group)

Hematological change	Untreated control	Cadmium	Cadmium/whey protein
Red blood cells count (10^6/mm^3)	5.71 ± 0.08	4.21* ± 0.13	5.53** ± 0.02
Total leucocytic Count (10^3/mm^3)	6.87 ± 0.41	5.47* ± 0.29	6.40** ± 0.04
Hemoglobin concentration (g/dL)	13.27 ± 0.39	9.86* ± 0.37	12.69** ± 0.45
Packed cell volume (%)	46.14 ± 0.33	37.18* ± 0.61	43.82** ± 0.73

The shown values in the table are means ± S.D. * Means with significant difference ($P < 0.05$) from control, ** means with significant difference from that of cadmium-treated animals.

Concerning the estimated haematological parameters, animals exposed to cadmium and administered with whey protein (group 3) demonstrated no significant differences concerning the untreated control animals.

As regards the biochemical changes, total proteins, albumin, and globulin were decreased in cadmium-treated animals that were not administered with whey protein (group 2). Animals of this group demonstrated a significant increase in the levels of ALT, ALP, AST, urea, creatinine, BUN and bilirubin. Levels of total thiols, glutathione, TAC and catalase (serum and tissues) were decreased in the group of rats exposed to cadmium and had no access to whey protein. Animals in this group exhibited a significant increase in the levels of MDA and H_2O_2 concerning that of the control levels.

The cadmium-exposed animals that were given whey protein (group 3) exhibited significantly decreased levels of ALP, ALT and AST, and a significant increase in the levels of total thiols, glutathione, TAC and catalase (serum and tissues)

compared to group 2 animals. These animals also disclosed a significant decrease in the levels of H_2O_2 and MDA compared to cadmium-exposed animals (group 2). The improvements in biochemical profile shifted the altered levels toward the control levels.

Concerning the estimated biochemical parameters, animals exposed to cadmium and administered with whey protein (group 3) demonstrated no significant differences concerning the untreated control animals.

Table (2 a, b, c, d) shows the biochemical changes in rats exposed to cadmium, and rats exposed to cadmium and administered with whey protein compared to the untreated control rats.

Table 2: Biochemical changes in rats exposed to cadmium, and rats exposed to cadmium and administered with whey protein compared to the untreated control rats (n= 20 animals/group).
Table 2a: The estimated serum levels of total proteins, albumin, globulin, and bilirubin in the animals of various groups

Parameter	Control	Cadmium	Cadmium/whey protein
Total proteins (g/dL)	7.91 ± 0.11	5.88* ± 0.14	7.52** ± 0.13
Albumin (g/dL)	3.51 ± 0.05	2.40* ± 0.13	3.30** ± 0.15
Globulin (g/dL)	3.96 ± 0.02	2.78* ± 0.13	3.57** ± 0.27
Bilirubin (mg/dL)	6.74 ± 0.34	10.18* ± 0.23	8.89** ± 0.48

The shown values in the table are means ± S.D. * Means with significant difference ($P < 0.05$) from control, ** means with significant difference from that of cadmium-exposed animals.

Table 2b: The estimated serum levels of alanine transferase (ALT), aspartate transferase (AST), and alkaline phosphatase (ALP) in the animals of various groups

Parameter	Control	Cadmium	Cadmium/whey protein
(IU/L)	27.87 ± 1.02	56.11* ± 1.19	33.61** ± 1.05
AST (IU/L)	42.11 ± 1.07	117.14* ± 3.47	57.17** ± 1.14
ALP (IU/L)	24.67 ± 1.16	68.73* ± 1.49	30.89** ± 1.67

The shown values in the table are means ± S.D. * Means with significant difference ($P < 0.05$) from control, ** means with significant difference from that of cadmium-exposed animals.

Table 2c: The estimated serum levels of creatinine, blood urea nitrogen (BUN), and urea in the animals of various groups

Parameter	Control	Cadmium	Cadmium/whey protein
Creatinine (mg/dL)	0.58 ± 0.01	0.84* ± 0.05	0.66** ± 0.31
BUN (mg/dL)	16.31 ± 1.09	23.46* ± 1.41	17.83 ± 1.53
Urea (mg/dL)	40.02 ± 0.71	69.08* ± 0.68	47.19** ± 0.73

The shown values in the table are means ± S.D. * Means with significant difference ($P < 0.05$) from control, ** means with significant difference from that of cadmium-exposed animals.

Table 2d: The estimated serum levels of total thiols (mmol/L), glutathione (GSH) (µg/mL), catalase (IU/L), total antioxidant capacity (TAC) (nmol/mL), hydrogen peroxide (H2O2) (mmol/L), and malondialdehyde (MDA) (nmol/mL) in the animals of various groups

Parameter	Control	Cadmium	Cadmium/whey protein
Total thiols (mmol/L)	2.48 ± 0.42	0.29* ± 0.06	1.97** ± 0.46
Glutathione (µg/mL)	41.23 ± 1.14	16.03* ± 0.61	37.14** ± 1.35
Catalase (IU/L)	53.42 ± 1.63	31.17* ± 1.05	47.19**± 1.28
TAC (nmol/mL)	36.39 ± 1.06	15.44* ± 1.06	28.34** ± 1.08
H_2O_2 (mmol/L)	40.73 ± 1.52	91.81* ± 1.13	51. 13** ± 1.05
MDA (nmol/mL)	324.17 ± 3.11	420.31* ± 3.52	349.37** ± 3.61

The shown values in the table are means ± S.D. * Means with significant difference (P < 0.05) from control, ** means with significant difference from that of cadmium-exposed animals.

Table (3) shows the levels of glutathione, total thiols, catalase, TAC, H_2O_2, and MDA in the liver and kidney homogenates of rats exposed to cadmium, and rats exposed to cadmium and administered with whey protein compared to the untreated control rats.

Table 3: The estimated total thiols (mmol/L), glutathione (µg/mL), catalase (IU/L), malondialdehyde (MDA) (nmol/mL), H_2O_2 (mmol/L) and total antioxidant capacity (TAC) (nmol/mL) in the liver and kidney homogenates of animals in the various groups

Parameter	Untreated Control		Cadmium		Cadmium/whey protein	
	Liver	Kidney	Liver	Kidney	Liver	Kidney
Total thiols (mmol/L)	1.81 ± 0.26	1.07 ± 0.16	0.21*±0.05	0.19*±0.04	1.64** ± 0.35	1.79** ± 0.35
Glutathione (µg/mL)	16.40 ± 1.13	14.66± 1.03	5.13*±0.51	4.83*±0.69	14.44**± 1.29	14.63**± 1.24
Catalase (IU/L)	20.12 ± 1.52	19.07 ±1.11	9.08*±1.06	9.11*±1.07	17.56**± 1.21	17.61**± 1.46
MDA (nmol/mL)	124.06± 3.18	120.31± 3.65	416.21*±3.14	485.21*±3.64	145.18**±3.41	146.08**±3.31
H_2O_2 (mmol/L)	16.49 ± 1.49	13.34 ± 1.23	87.81*± 1.41	95.21* ± 1.62	18.86**± 1.11	17.83**± 1.05
TAC (nmol/mL)	14.28 ± 1.12	13.61 ± 1.19	6.25* ± 1.22	5.91* ± 1.05	12.45**±1.06	12.77**±1.08

The shown values in the table are means ± S.D. * Means with significant difference (P < 0.05) from control, ** means with significant difference from that of cadmium-exposed animals.

Discussion

A limited amount of free radicals is required to carry out some physiological metabolic activities such as detoxification and cell respiration. Free radicals involving hydroxyl and superoxide radicals, and non-radicals such as hydrogen peroxide are grouped under reactive oxygen species (ROS).[29] Excess amounts of ROS, encompassing free radicals, are highly oxidizing to cell molecules and create a state of oxidative stress (-pronounced oxidative damage) which affects cell proteins, DNA and even some genes.[2,30,31] Lipid peroxidation, mainly affecting

cell and organelle membranes, is among the most deleterious effects provoked by ROS.[32]

The highly potent endogenous antioxidant system, existing in human and animals, is a highly sensitive detector to excess reactive oxygen species and acts to prevent and/or block their deleterious actions.[2,4,5-7,33] Superoxide dismutase, catalase and glutathione peroxidase are endogenous antioxidant enzymes, while Metallothioneins are examples of non-enzymatic antioxidants.[34-36] All these endogenous antioxidant bioactive molecules contribute, via different mechanisms, to the elimination of excess free radicals.[22,25, 37]

The endogenous antioxidant mechanisms may be impaired in case of heavy metals toxicity; this occurs under the effect of the overwhelming oxidative stress that depletes the active antioxidant molecules. In other words, the effect and magnitude of the oxidative molecules, exemplified by free radicals, exceed the counteracting action of the antioxidative molecules.[2] In such cases, an external additive source of antioxidants is required, preferably in the form of additive supplementary nutrients, to restore the oxidation: antioxidation balance. The antioxidative activity of these nutrients is proposed to support the endogenous antioxidation capacity in various ways. One of these ways is the prevention of lipid peroxidation which is one of the main functions of potent antioxidants.[12]

If proteins are selected as additive nutrients, then the main role of these external natural proteins is to function as efficient antioxidants, synergistically with the endogenous antioxidant system, to inhibit lipid peroxidation and thus prevent cell damage. This is accomplished through the elimination (scavenging) of free radicals and binding (chelation) with pro-oxidative molecules, as well as enhancing the reduction of reactive oxygen species.[14,29]

Cadmium, as a highly toxic heavy metal, was employed in the current study to induce a state of oxidative stress in rats. After absorption, cadmium is distributed to different tissues and organs and starts to accumulate especially in the liver and kidney.[1] The accumulated cadmium induces oxidative damage in these organs through the indirect generation of free radicals.[38] Induction of oxidative stress and direct action of cadmium on lipid peroxidation of cell membranes are the main events in the progression of cadmium cytotoxicity.[39] Lipid peroxidation of mitochondrial membranes drastically affects ATP production and is associated with a sharp decrease in mitochondrial glutathione.[40] Moreover, cadmium exerts a suppressive effect on antioxidative enzymes and thus accentuates the already existing oxidative stress. Finally, apoptosis takes place in cadmium toxicity because of caspase activation.[41]

The presently encountered elevations of serum enzymes ALT, AST and ALP reflect the cadmium-induced hepatic damage. Increased serum levels of hepatic enzymes are strongly linked with hepatotoxicity.[42] The damaged lysosomal membranes due to lipid peroxidation become more permeable with leakage of hepatic enzymes and subsequent increase of their serum levels. In addition, biochemical parameters indicating kidney functions, including urea and creatinine, were increased in the presently cadmium-intoxicated rats. These point to a nephrotoxic effect and impaired renal functions caused by cadmium toxicity. Metallothionein, especially in hepatic tissues, is known to contribute to antioxidation activities through its property to bind the pro-oxidative metals.[27]

Cadmium-induced nephrotoxicity is presumably ascribed to the released cadmium-metallothionein complexes originating from the damaged hepatic tissue.

The presently estimated levels of total thiols, glutathione, TAC and catalase, which reflect the oxidative stress and the antioxidative status, were all decreased in rats exposed to cadmium. The oxidation activity of ROS, generated by toxicities, extends to involve most such antioxidant molecules that may lose their activity. Glutathione and catalase are actively incorporated in scavenging free radicals, and catalase acts as a potent antioxidant by destructing hydrogen peroxide and thus eliminating the major cause of lipid peroxidation.[30] Depletion of these endogenous antioxidant molecules allows free radicals to provoke more intensive lipid peroxidation.[43] Additionally, in the presence of oxidative stress, glutathione-related proteins may be converted to reduced forms.[44] This may interpret the decreased glutathione level in rats currently exposed to cadmium. The level of total thiols was also decreased in the presently cadmium-intoxicated rats.

Thiols, involving glutathione, under oxidative stress may undergo oxidation and their role in scavenging singlet oxygen and hydroxyl radicals is minimized.[45] The presently encountered increased level of MDA is a direct expression of the existing oxidative damage, since MDA is generated because of lipid peroxidation of the cellular membranous structures.

It has been stated that dietary antioxidants help protect against cell damage induced by excess free radicals and thus ameliorate the effects of oxidative stress.[14] In the present study, cadmium blood level was significantly decreased in rats intoxicated with cadmium and given whey proteins compared to rats exposed to cadmium. This might be ascribed to a postulated indirect chelating activity of whey proteins through enhancing the formation of cadmium complexes with metal-binding proteins. Undoubtedly, minimizing the blood cadmium pool, which represents the initial insult, reduces greatly the eventual power of oxidative stress. This subsequently gives the chance to the endogenous antioxidant system to relatively recover and counteract the effects of excess free radicals.

Conclusion

In the presence of the postulated antioxidant role of whey proteins, which act synergistically with the endogenous antioxidant molecules, the overall antioxidant status was much improved. This was evidenced by the improved TAC in the animals exposed to cadmium and administered with whey protein. Measurement of TAC is indicative of counteracting oxidative stress, i.e., the enhanced TAC is associated with alleviation of cell damage induced by oxidative stress. This finally reflected in the haematological and biochemical profiles of rats intoxicated with cadmium and given whey proteins. The estimated parameters were improved and shifted toward the control levels. This might be considered evidence of the antioxidative activity of whey proteins against the tremendous oxidative damage induced by cadmium toxicity.

As a limitation of the present study, the cadmium level was not measured in liver and kidney tissues to provide direct evidence of the cadmium-induced damage in these tissues.

More investigations are recommended to define the detailed molecular mechanisms relevant to the antioxidative role of supplementary proteins in case of toxicities. In addition, future experimental work should focus on the measurement of cadmium in the hepatic and renal tissues to clarify the direct cadmium-induced toxic effects.

Authors' Contribution

M Z, H R, and AAD performed the laboratory work. MAE supervised the experiment. NHA, KNY, EAA, SA, and FAN were responsible for the lab work, software, and statistical analysis of data. M M designed the study, supervised the methodology and wrote the manuscript.

Ethics Approval

The guidelines for the care and use of laboratory animals, per the institutional and national regulations, stated by the "Research Ethics Committee" of Imam Mohammad Ibn Saud Islamic University (IMSIU) were accurately followed (LAB-animals-022-0198).

Availability of Data

Data will be made available on request

Conflict of Interest

The authors declare that they have no relevant financial or non-financial interests to disclose.

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