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Research Article

Effects on Bodyweight, Organ Weight and Kidney Function of Sub-Chronic Fluoride Exposure, and the Role of *Moringa stenopetala* Extract in Mice

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Abstract

Fluoride in excess is a toxic chemical to the kidney function. Studies implicated that aggravating oxidative stress and inhibiting body antioxidative systems is the main route of damage. *Moringa stenopetala* is rich in antioxidants and has a potent anti-oxidative effect. The study aimed to determine the effect of sub-chronic exposure to fluoride and the effect of *Moringa stenopetala* crude extract on body weight and kidney function. Randomized control study was conducted using 2 months old, 24 adult Swiss Albino mice. All animals were allowed free access to standard pellet diet ad libitum and distilled water. The mice were randomly divided into six groups of four animals, taking different doses of fluoride and the crude extract. After 3 months of exposure, the mice were anesthetized and sacrificed under anesthesia for sample collection. There was a statistically significant difference in renal function test (plasma urea) between the treated and the control groups in high dose fluoride group. Independent t-test yields no significant difference body mass and organ mass between fluorotic groups without *Moringa stenopetala* and fluorotic with *Moringa stenopetala* groups (P = 0.318). Though, there was some evidence indicating *Moringa stenopetala* might provide some protection, further studies are necessary to explore the role of *Moringa stenopetala*, as no significant difference was observed between groups with and without the plant extract in this study.

Keywords: *Fluorosis; Sodium Fluoride; Renal Function; Moringa stenopetala; Weight; Mice*

Background

Several sources of drinking water specially ground water in certain parts of the world including Ethiopia are contaminated with high fluoride due to the geographical distribution of the element and its compounds [1,2]. Fluoride in low concentration have protective effects on dental caries and generally for bone health. Considering these benefits fluoridation of drinking water is recommended in areas where naturally occurring fluoride concentration is lower than the recommended levels [3]. Although fluoride ingestion is useful to a certain extent for bone and teeth development, chronic over intake of fluoride is known to cause fluorosis. World Health Organization (WHO) permits only 0.5 - 1.5 mg L^{-1}as a safe limit of fluoride in drinking water as cited in Viswanathan G., *et al*. 2009 [4]. Skeletal and dental toxicity was most commonly occurring adverse effects of fluorides; however, soft tissues were also afflicted with damage by F [5,6]. Soft tissues are also affected by the excess F, which is referred to as non-skeletal fluorosis, the kidney is among the organs affected by excessive fluoride intake [7].

Kidney is the main excreting organ of excessive fluorides in the living system. Approximately 85% of fluorine in the body is excreted through kidney. Fluoride is a toxic chemical to the kidney and can damage renal tubular cells with morphological alterations as evidenced by previous findings [8]. Fluoride even in lower concentrations can induce a significant tubular disfunction, resulting in dilute urine, impaired protein reabsorption, and increased calcium and phosphate urinary excretion [9].

There were various suggested molecular mechanisms in fluoride toxicity. As fluoride have highly chemical property, can directly attack oxygen and disturbs oxygen metabolism after exposed to excessive amounts of fluoride in the living system [10]. Generation of oxygen free radical and depletion of the body antioxidant system is believed to be the main mechanism in fluoride toxicity [11]. Other possible mechanisms include damage to cells secondary to calcium retention and apoptosis [12,13].

Moringa (Moringa stenopetala, Bac.) a tree that belongs to the family Moringaceae is indigenous and cultivated in Ethiopia [14]. Apart from being consumed as a vegetable *M. stenopetala* is also used to treat malaria, hypertension, stomach disorder and dysentery, asthma, diabetes, epilepsy and visceral leishmaniosis by local communities [15].

The *M. stenopetala* extract is rich in antioxidants including rutin and has potent anti-oxidative effect [16,17]. The leaves are highly nutritious, being a good source of protein, β-carotene, vitamins A, B, C and E, riboflavin, nicotinic acid, folic acid, pyridoxine, amino acids, minerals and various phenolic compounds [18,19]. The contents of this plant have antioxidants capacity and other like vitamins participate with enzymes having antioxidant activity.

Aim of the Study

The present study aimed to investigate the effect of fluoride exposure on kidney histology and function. The protective effect of this indigenous plant extract against histological and functional alterations was investigated.

Materials and Methods

Animals: The study was conducted using laboratory-bred swiss albino Mice. Twenty-four (12 male and 12 female) apparently healthy, 7 - 8 weeks old mice weighing 33.8 ± 5.5g, were collected from Tropical and Infectious Disease Research Center, Jimma University. The animals were housed in rectangular polypropylene cages (four mice per cage in order to provide ample spaces for movement and ambient temperature) with sawdust as bedding material and to soak the excretory fluids. Throughout the study period, the male and female mice were kept in separate cages to avoid breeding and maintained under constant laboratory conditions of temperature (22 ± 2°C) with 12 hours light/dark cycle. All animals were allowed free access to standard pellet diet ad libitum and distilled water except when starvation was otherwise needed. An acclimatization period of 7 days was allowed before experimentation, in order to minimize any non-specific stress as suggested by different scholars in various similar studies.

Fluoride preparation: NaF was used to sub-chronically expose the mice to fluoride. NaF with 98.5% purity was purchased from LOBA Chemie laboratory reagents and fine chemicals Mumbai, India. A stock solution of 2000 ppm fluoride was prepared by dissolving 4.42g of NaF in 1L of distilled water. The stock solution was diluted with distilled water to prepare different test doses of fluoride at concentrations of 0.07 ppm, 60 ppm and 100 ppm. To prepare working solution with 0.07 ppm fluoride, 0.035 ml of the stock solution was diluted by 1L of distilled water; 60 ppm fluoride: 30 ml of stock solution diluted in 1L distilled water; and 100 ppm fluoride was obtained by diluting 50 ml of stock solution by 1L distilled water. New batches of test solutions, not older than one week were used for administration [23].

Preparation of *Moringa stenopetala* aqueous extract: The fresh leaves of *Moringa stenopetala* were collected from local garden and confirmed by experts. The leaves were rinsed with tap water to clean off extraneous materials and air-dried under shade without sunshine exposure in shadow room, then powdered. The dried leaves were then cut into pieces and ground to a fine powder using a sample

mill. The powdered substance was then weighed using an analytical balance and stored at room temperature until extraction. One hundred gram of the powdered plant material was soaked in 1000 ml of distilled water in the conical flask at room temperature and stirred intermittently for 72 hours. The material will then be filtered using sterile Whatman No.1, 15 cm filter paper into a clean conical flask. The filtrates were freeze-dried in a lyophilizer to yield a crude extract. The crude extract was kept in desiccators at -20° C until used.

Design: Experimental (Randomized Controlled study) were used. These 24 mice randomly allocated into six groups, the control and experimental groups took different doses of fluoride and *Moringa stenopetala* alone, or together as indicated in table 1. The sample size was adequate for analysis in this study as one way ANOVA was used [20].

Groups (N = 4)	1	2	3	4	5	6
Fluoride Dose (ppm)	Control	60	100	60	100	0.07
Moringa Treated (mg/Kg)	-	-	-	100	100	100

Table 1: *Grouping and dosage of fluoride and Moringa stenopetala.*

At the end of the experiment, 12 hours before the Euthanasia animals deprived of food and drink. After fasting, the animals were humanely sacrificed under anesthesia. Immediately after anesthesia, the blood sample was collected via cardiac puncture. The serum samples of mice were investigated to assess Urea, Creatinine, and BUN levels using automated clinical chemistry machine Human star 100 [26].

Statistical analysis: After data collection and cleaning, data entered into SPSS software version 20 for statistical analysis; results expressed as mean ± SD. Statistical data analysis was performed using a paired t-test, independent t-test and one-way ANOVA with post hoc Bonferroni test, where $p \leq 0.05$ considered statistically significant.

Ethical issues: In the administration of substances, appropriate techniques in a professional manner, enabling the achievement of the anticipated results whilst causing minimum distress to the animals were applied. The physicochemical properties of administered substances and their vehicle were in accordance with the biocompatibility criteria for the route of administration: local tolerance, temperature, sterility, osmolality, in order to guarantee the prevention of side effects such as irritation and traumatization during administration.

To minimize the study animals suffering humane procedures followed in handling the animals and during sample collection and euthanasia. Enriching of the cages in which the animals will be kept, using anesthesia during sample collection and scarification of the study animals will be among the steps to decrease the distress and suffering. Disposal of contaminated laboratory utensils, animal's tissues and waste chemicals handled according to laboratory safety guidelines. Ethical clearance was obtained from Jimma University Institutional Review Board.

Result

The initial mean weight for different treatment groups and control group is shown in table 2. There was no significant difference between weight of groups ($p > 0.05$) before the commencement of the study. But there was a gradual increase in body weight of mice in then after given in table 3. Mice in group 3 had a gradual weight decrement. The percentage of weight increment or decrement (calculated using the formula below) is different for different treatment and control groups, according to the initial weight; the control group has 54.62% weight increment. Mice with 60 ppm fluoride without *Moringa stenopetala* show slight decrement of weight which is 2.79%. The higher dose of all groups was 100ppm fluoride without *Moringa stenopetala* had the pronounced decrement in weight in all groups by 27.06%. There was slight increment of weight in group 4 (60 ppm+ 100 mg/Kg *Moringa stenopetala*) and group 5 (100 ppm + 100 mg/Kg *Moringa stenopetala*) 12.9% and 0.7% respectively. Group 6 took 100 mg/Kg *Moringa stenopetala* crude extract without fluoride shows increment in weight by 28.96%.

Group (N = 4)	1	2	3	4	5	6
Initial	29.75 ± 7.22	37.75 ± 4.7	33.25 ± 5.5	31 ± 5.2	35.25 ± 4.2	36.25 ± 4.1

Table 2: *Body weights of mice in gram) initially showing there was no significant difference between groups ($p > 0.05$).*

Group (N = 4)	1	2	3	4	5	6
Initial	29.75 ± 7.22	37.75 ± 4.7	33.25 ± 5.5	31 ± 5.2	35.25 ± 4.2	36.25 ± 4.1
15th day	33 ± 7.44	37 ± 5.22	35.75 ± 4.35	32.5 ± 7	34 ± 5.47	36.75 ± 5.44
30th day	36 ± 7.43	37.25 ± 5.90	34 ± 3.46	33 ± 5.71	34.75 ± 5.37	39 ± 5.65
45th day	36.5 ± 7.93	38 ± 6.88	30.25 ± 2.50	34 ± 5.22	35.5 ± 5	40.5 ± 3.69
60th day	39.25 ± 8.09	38.25 ± 6.60	31 ± 4.54	34.75 ± 4.57	35.25 ± 5.12	42.5 ± 2.38
75th day	41.5 ± 6.85	37.75 ± 6.18	27 ± 4.69	35.5 ± 5.44	34.25 ± 5.62	44.75 ± 2.75
Final	46 ± 8.08	36.5 ± 5.2	24.25 ± 4.1	35 ± 4.3	35 ± 5.2	46.75 ± 2.2

Table 3: *Body weights of mice initially and after treatment, in gram, with fluoride and Moringa stenopetala crude extract. There was statistically significance difference in body weight gain between the treated and the control groups. Compared with the controls, weight of 100 ppm fluoride group had significant weight decrement at 10th and 12th weeks of treatment with $F (5, 23) = 5.19$, $p = 0.02$, $\eta^2 = 0.59$ and $F (5, 23) = 10.25$, $p = 0.000$, $\eta^2 = 0.74$ respectively. Paired t test for final and initial weights were performed and hence, there was no significant difference ($p = 0.072$), independent t test also yields no significance difference between two fluorotic groups without Moringa stenopetala (group 2 and 3), and two fluorotic groups with Moringa stenopetala (group 4 and 5) ($P = 0.17$), but there was significant difference between group 3 (100 ppm) and group 5 (100 ppm and Moringa stenopetala extract) $t(6) = -3.208$. $P = 0.018$. Where, F = F statistics in one-way ANOVA, P = Level of significance, η^2 = Effect size, df = Degree of freedom.*

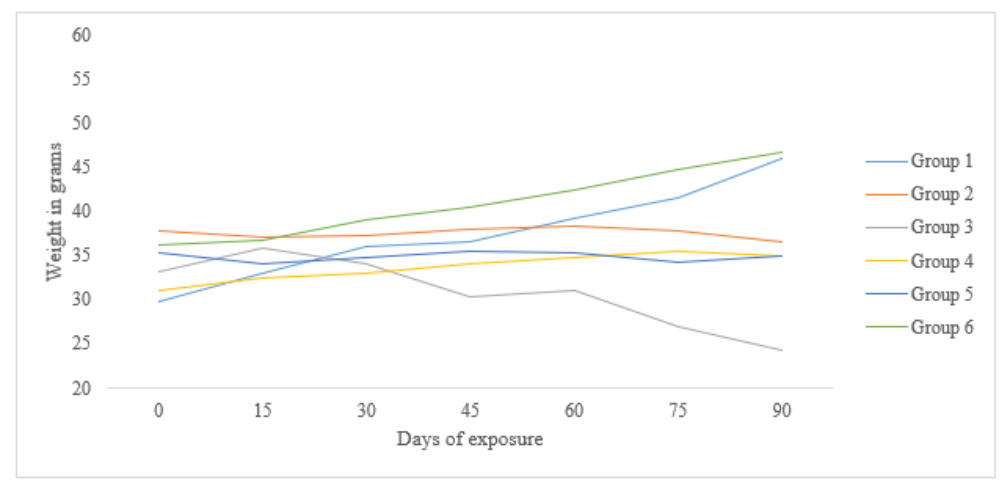

Figure 1: *Body weight increasing/decreasing trends in control and different experimental groups of mice.*

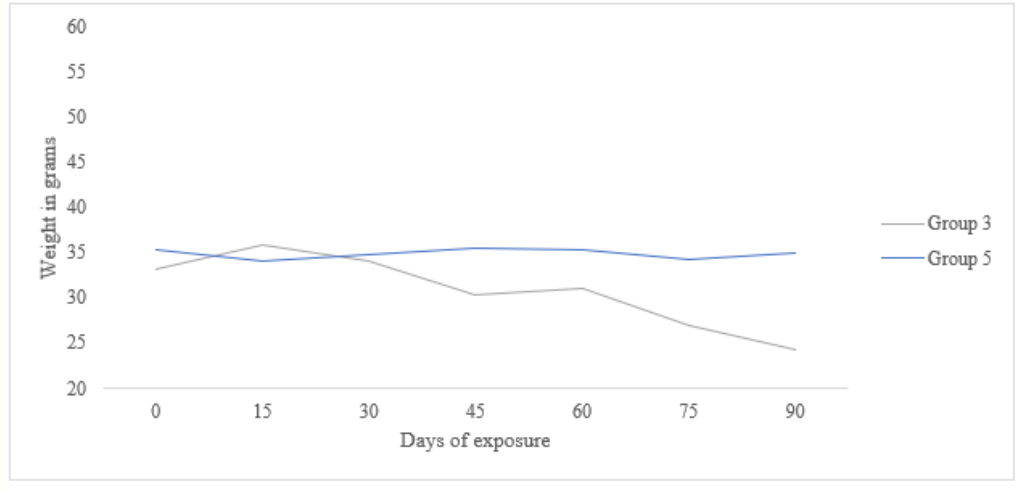

Figure 2: *Weight trends in high dose fluoride groups with and without Moringa stenopetala showing significant final weight difference.*

The subchronic effect of fluoride and aqueous extracts of the plant on organ weight of mice were illustrated in table 4. The mean absolute weights of brain and thyroid gland showed decrement as compared to control mice, but the decrement in organs weight were statistically insignificant.

Group	Heart	Brain	Thyroid	Liver	Kidney	Pancreas
1	.267 ± .077	.469 ± .049	.0278 ± .012	3.17 ± .903	.341 ± .092	.325 ± .040
2	.263 ± .1303	.466 ± .030	.0157 ± .001	2.85 ± .471	.423 ± .054	.329 ± .092
3	.209 ± .0786	.437 ± .038	.0138 ± .002	2.86 ± .725	.317 ± .124	.429 ± .194
4	.203 ± .0681	.440 ± .049	.0149 ± .002	2.084 ± .582	.289 ± .119	.233 ± .043
5	.1675 ± .065	.377 ± .028	.014 ± .002	1.491 ± .858	.228 ± .087	.225 ± .178
6	.246 ± .0174	.440 ± .032	.02 ± .006	3.11 ± 1.29	.393 ± .091	.35 ± .079

Table 4: *Organ weight of mice (in gram) after treatment with fluoride and Moringa stenopetala extract*

The mean and standard deviation of renal function tests of treatment and control group is given in table 5. Creatinine level of only fluoride taking group was increased compared with the control and both fluoride and *Moringa stenopetala* taking groups. Plasma urea concentration had a similar trend where, increment was observed as the dose of fluoride increased. *Moringa stenopetala* has shown to decrease this rise in plasma urea level. The concentration of BUN seems steady in all groups except the high fluoride taking group (3), as comparable results were recorded otherwise.

Group	Creatinine (mg/dl)	Urea (mg/dl)	BUN (mg/dl)
1	0.136 ± 0.020	52.60 ± 1.30	25.96 ± 2.18
2	0.143 ± 0.020	55.16 ± 8.72	23.86 ± 2.61
3	0.233 ± 0.120	103.90 ± 45.06*	42.22 ± 24.41
4	0.133 ± 0.011	46.33 ± 8.96	28.50 ± 15.91
5	0.120 ± 0.00	50.63 ± 13.84	23.66 ± 6.46
6	0.120 ± 0.017	37.10 ± 10.74	17.33 ± 5.01

Table 5: *Renal function result after treatment with fluoride and M. stenopetala extract for subchronic toxicity study in mice (Mean ± SD).*

**P < 0.05.*

After checking the assumptions (skewness < |2| kurtosis <|9|, P = 0.656 after Levene's Test of Equality of Variances) independent students t-test, paired t-test and one-way ANOVA followed by post hoc Bonferroni test was performed for renal function parameters. In this study, the serum levels of urea, BUN, and Creatinine in the control mice were in the normal range of healthy mice; mean and standard deviation were given in table 5.

There was a statistically significant difference in concentration of plasma urea between the treated and the control groups. Compared with the control, 100 ppm fluoride without *Moringa* treated group (group 3) had a significant increase in plasma urea level with F (5 17) = 3.966, P = 0.023, η = 0.625 and power of 0.801.

Although there were increment trends in plasma creatinine and BUN levels, there was no significant difference between control group and different experimental groups in both cases. To evaluate the role of *Moringa stenopetala*, independent t-test was performed between non-*Moringa* group (group 1 - 3) and *Moringa* group (group 4 - 6), still yields no significant difference.

Discussion

At the end of the experiment, the gain in body weight in all groups treated with NaF without *Moringa* was lower than those groups co-treatment NaF with *Moringa* which were similar to other finding by Samal U., *et al*. (1994) in which they reported that a decrease in mean body weight of both sexes was recorded in toads from the fluoride contaminated area [21]. In laboratory studies, growth retardation has been observed in rats maintained for 6 months on a diet with a fluoride content of 3 mg/kg and drinking water with 100 mg/liter fluoride [22]. In contrast to our finding the finding by Zhan X., *et al*. reported tha administration of 100 ppm of fluoride for 6 weeks starting in 3-month-old rats did not cause significant changes in body weight compared to controls [23]. The difference might be due to short treatment period in their study since effect of fluoride is dose dependent and duration dependent.

Our study results showing significant differences in body weight among the groups during 10^{th} and 12^{th} weeks were in contrast with some previous studies. In the study of Collins., *et al*. [24], male and female rats exposed to fluoride for 10 weeks at levels up to 175 ppm in drinking water did not show any significant differences in body weight gain among groups. Mice exposed to 100 ppm fluoride in drinking water without *Moringa* showed a significant decrease in body weight gain compared to the controls. Relative organ weights were also not affected in this study.

The exposure to sodium fluoride in albino rats orally administrated with different doses for 15 days of duration showed the body weight of treated rats gain initially for 1 or 2 days and after that decreases significantly as compared with control rats [25]. According to the study by Tsunoda M., *et al*. oral administration of NaF led to a significant reduction in body weight gain [26].

Metabolic, functional and structural damage caused by sub chronic fluorosis have been reported in many tissues. Acute fluoride poisoning in the sheep has been associated with hypoglycemia. It has been suggested that the hypoglycemia is caused by blockade of the production of propionate and amino acids by fluoride [27]. These substances are converted to glucose through the Krebs cycle and are the main source of glucose.

Apart from this, the blockage of Krebs cycle may well verify by a significant decline in epididymis succinate dehydrogenase (SDH) in NaF treated mice suggest a blockage in the conversion of succinate to fumarate. The mitochondrial structure and function are also likely to be disturbed, since SDH is a mitochondrial enzyme, and therefore oxidative metabolism will suffer [28]. Research data strongly suggest that fluoride inhibits protein secretion and/or synthesis leading to weight reduction [29].

The significant decline in epididymal succinate dehydrogenase (SDH) in NaF treated mice suggests a block in Krebs cycle in the conversion of succinate to fumarate. The mitochondrial structure and function are also likely to be disturbed, since SDH is a mitochondrial enzyme, and therefore oxidative metabolism will suffer [30-33]. Research data strongly suggest that fluoride inhibits protein secretion and/or synthesis and that it influences distinct signaling pathways involved in proliferation and apoptosis including the mitogen-activated protein kinase (MAPK), p53, activator protein-1 (AP-1) and nuclear factor kappa B (NFB) pathways [34,35].

In the present investigation, an increase in the urea level suggests impairment in glomerular function induced by NaF. The elevated serum levels of BUN indicated the reduced ability of the kidney to eliminate the toxic metabolic substances [36,37]. F is excreted mainly from the kidney, and harmful effects of F retention are directly related to renal function. Fluoride disturbed the kidney filtration and renal function by significantly increased the level of urea in plasma [38,39].

This study agreed with previous studies which have demonstrated that NaF treatment of rats causes alterations in the serum biochemical parameters including an altered renal function [40]. Because the concentration of Creatinine in blood correlates inversely with the volume of glomerular filtration, the former is a useful marker of the filtration function of kidneys, the more so in that Creatinine is excreted only via the kidneys [41]. The impaired renal function would also explain the drastic increase of the area in the blood serum of the treated mice. A lower rate of secretion of urea into urine resulting from renal insufficiency would cause its concentration in serum to increase. Appleton., *et al*. (2000) after injection of high doses of sodium fluoride into rats found increases in the concentration of glucose, urea, and Creatinine in the plasma. The increase of urea and Creatinine concentration in the serum was interpreted by this author as reflecting impaired renal function [42]. Renal lesions caused by the presence of fluorides have already been reported by numerous authors [43,44].

According the finding by Omireeni N. and Siddiqi A (2010) higher doses of sodium fluoride 10, 20 and 30 mg of NaF/kg body weight caused a significant decrease in the collagen content of the kidneys when compared to the control rats which was may be either due to increased degradation of collagen or an inhibition of collagen synthesis by NaF [36].

These authors added that the collagen fibers produced during fluoride toxicity would be defective due to inadequate cross-links. Thus, fluoride interferes with the maturation and normal metabolism of tissue collagen. The glomerulus is the functional unit of the kidney. The normal glomerular basement membrane, composed of type IV collagen, plays an important function in the process of filtration [36].

These histological changes could be correlated with oxidative damage. *Moringa stenopetala* crude extract had attenuated these fluoride-induced pathological changes in liver and kidney tissue of mice since *Moringa* is very rich in antioxidants substance including Rutin. The presence of *Moringa* alleviated the harmful effects of fluoride, such as mitigating pathomorphological changes of liver and kidney. The antioxidant activities and health benefits of *Moringa* may be related to its high rutin and phenolic content. Rutin has shown a higher antioxidant activity than vitamins C and E. These compounds are able to capture free radicals by donation of phenolic hydrogen atoms [45].

Conclusion and Recommendations

From the study, fluoride was found nephrotoxic chemical caused renal nephron damage that led to increased plasma urea level, while crude extract of *Moringa stenopetala* provided some ameliorative effect on renal urea clearance. While we can conclude about the toxic effects of fluoride on the kidney, we can't be sure about the positive findings of *Moringa stenopetala*, as no significant difference was observed between groups with and without the plant extract. As it is promising in many prospects, the plant extracts needs to be evaluated with different extraction solvents and different dose adjustments.

Author Contributions

Authors have approved the submitted version and agrees to be personally accountable for this journal. Conceptualization, M.I. S and A.D.W.; methodology and report writing, A.A.T. and M.B.G.; data curation and sample analysis, A.A.T., M.B.G., T.A.N., M.Bz and D.T; Funding acquisition, M.I.S.; reviewing, editing and supervision, M.I.S and T.G.

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Conflicts of Interest

The authors declare no conflict of interest.

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