The Mundy Firm PLLC

4131 Spicewood Springs Rd, Suite O3
Austin, Texas 78759
512-334-4300
E-Mail: jeff@jmundy.com

April 8, 2020

Via U.S. Certified Mail/RRR

Permian Highway Pipeline, LLC
1001 Louisiana St. Ste. 1000
Houston, Texas 77002-5089

Via U.S. Certified Mail/RRR

Kinder Morgan Texas Pipeline LLC
1001 Louisiana St. Ste. 1000
Houston, Texas 77002-5089

RE: Notice of Intent to Sue Permian Highway Pipeline, LLC and Kinder Morgan Texas Pipeline LLC for Violations of the Safe Drinking Water Act, the Clean Water Act, and the Resource, Conservation, and Recovery Act

To the Responsible Regulatory Agencies, and to Kinder Morgan and PHP,

Please be advised that Dr. Teri Albright, Dr. Milton Shaw, Ms. Paula Fowler, Mr. Max Fowler, and the Trinity Edwards Springs Protection Association (TESPA) on behalf of its members including the Wimberley Valley Watershed Association provide notice of their intent to file suit in federal court against Permian Highway Pipeline, LLC and Kinder Morgan Texas Pipeline LLC, hereafter both referred collectively as "Kinder Morgan" for violations:

1. of 42 U.S.C. §300h(b)(1)(A), the Safe Drinking Water Act, for unauthorized "injection activity in a manner that allows the movement of fluid containing any contaminant into underground sources of drinking water, [which] the presence of that contaminant may … adversely affect the health of persons." ;

2. of 42 U.S.C. § 6972(a)(1)(A) and (B), the Resource, Conservation, and Recovery Act commonly referred to as "RCRA", for creating an imminent and substantial endangerment to public health;

3. of 33 U.S.C. §§ 301, 402, and 404, the Clean Water Act, "CWA", for discharge of pollutants into waters of the United States in violation of the terms and conditions of

United States Army Corps of Engineers Nationwide Permit #12 and/or without a permit.

4. state law claims under the laws of the State of Texas including but not limited to nuisance, trespass, negligence, and gross negligence.

On or about March 28-29, 2020, personnel building the Permian Highway Pipeline in Blanco County, Texas, attempted to bore under the Blanco River in Blanco County, Texas. Rather than drilling a contained hole, they bored into the aquifer and released a plume of drilling fluid into the aquifer contaminating the sole source of well water for the area. The water wells of two homes approximately 1 and 1.5 miles away became filled with a cloudy/milkly contaminate within one day. The contamination persists. The homeowners report the cloudly/milkly discharge in the water leaves a greasy film on the kitchen sink and skin, which persists even using soap and scrubbing.

The size of the plume is unknown at this time. However, Kinder Morgan has acknowledged the milky discharge in the water is from the plume of drilling fluid from their boring activity. Kinder Morgan is the managing partner of the Permian Highway Pipeline project. The MSDS sheet provided by Kinder Morgan is attached.[1]

Please note that MSDS clearly and unequivocally warns the drilling "gel" is a Class 1a, human carcinogen. The MSDS does not specify which component is the carcinogen, although silica is a component and the International Agency for Research on Cancer, commonly known as "IARC", creator of the classification system, classifies silica as a Class 1, human carcinogen.[2] Bentonite, apparently the major constituent component of the AMC Gel is not a benign, inert material as Kinder Morgan is portraying in the public media. Attached as just a recent example is a study of Bentonite by Masoudi, et al., Journal of Toxicology & Industrial Health, Vol. 36, Issue 1, Feb. 25, 2020.

According to Kinder Morgan:

> *On Saturday, March 28, Permian Highway Pipeline (PHP) experienced an underground drilling fluid loss during construction in Blanco County, Texas. The drilling fluid is comprised of bentonite clay and water. Bentonite is a naturally occurring, non-hazardous, non-toxic clay. We strive for zero incidents and minimal environmental impact on all our construction projects. At this time, drilling operations have been suspended while the team evaluates the cause of the loss and determines the best path forward. We are working with affected landowners to address their needs. We are also consulting with our karst expert and the local water district manager to determine the best way to mitigate any current and future impacts. All of the appropriate regulatory agencies have been notified.*

[1] SDS for "AMC Gel" Safety Data Sheet, the Australian term analogous to our MSDS, Material Safety Data Sheet.

[2] https://monographs.iarc.fr/wp-content/uploads/2018/06/mono100C-14.pdf

The site of this discharge is the "disappearing" stretch of Blanco River at a location where the river water drains into the aquifer. This water flows into the aquifer and then moves back above surface into the Blanco River.

As a matter of law, the polluted water is an underground source of drinking water under the Safe Drinking Water Act, but also is a water of the United States under the Clean Water Act when as reappears in the Blanco River. This connectivity has been proven by hydrogeology dye trace studies, thus indicating that this discharge has impacted both an underground source of drinking water and a water of the United States.

Drilling in the geological region of the Blanco River Valley, Cypress Creek Watershed, and Wimberley Valley, where the route of the PHP pipeline is set to go is inherently rife with the potential of further events of this type to the point that some hydrogeologists see a recurrence of this pollution even to be a near certainty as this activity marches onward towards Wimberley, unless substantially changed and improved management practices are implemented.

These parties ask you as the guardians of the public water supply to please exercise your authority and discretion to protect these waters, which are the sole source of drinking water for an estimated 20,000 or more citizens in the Blanco River Valley. The parties will request the federal court to halt further construction of this pipeline and requests that Kinder Morgan work with TESPA and its hydrogeologists to find an alternative route that does not involve this type of risk to sole source aquifers and water supply reservoir for an even broader array of municipalities.

This notice is sent to you as required by the federal statutes under which Plaintiffs intend to proceed.

These citizen suit provisions include:

- Section 304 of the Clean Air Act (CAA);
- Section 505(a)(2) of the Clean Water Act (CWA);
- Section 1449(a)(2) of the Safe Drinking Water Act (SDWA);
- Section 11(g)(1)(A) of the Endangered Species Act (ESA);
- Section 105(g)(2)(A) of the Marine Protection, Research, and Sanctuaries Act (MPRSA, aka Ocean Dumping Act);
- Section 7002(a)(2) of the Resource Conservation and Recovery Act (RCRA);
- Section 20(a)(2) of the Toxic Substances Control Act (TSCA);
- Section 310(a)(2) of the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA); and
- Section 326(a)(1)(B) or (C) of the Emergency Planning and Community Right-to-Know Act (EPCRA).

Among the specific allegations for which TESPA will seek remedies in federal court include, but are not limited to:

1. violation of Section §300h(b)(1)(A) of the Safe Drinking Water Act by injecting fluids through a well into an underground source of drinking water without a permit. Sections 300j-8(a)(1)(A) and (B) provide that "any person may commence a civil action on his own behalf against any person…who is alleged to be in violation of any requirement prescribed by or under this subchapter."

2. violation of Section 7003 of the Resource Conservation and Recovery Act (RCRA) by disposing of solid waste into an underground source of drinking water, thereby creating an imminent and substantial endangerment to the public, *e.g*, it contaminated drinking water supplies; RCRA authorizes citizen suits to be brought for alleged violations of any permit, standard, regulation, condition, requirement, prohibition, or order effective pursuant to the Act. 42 U.S.C. § 6972(a)(1)(A). RCRA also authorizes citizen suits to be brought against any person who "has contributed to or who is contributing to the past or present handling, storage, treatment, transportation, or disposal of any solid or hazardous waste which may present an imminent and substantial endangerment to health or the environment[.]" 42 U.S.C. § 6972(a)(1)(B).

3. violation of Sections 301 and Section 404 of the Clean Water Act by violating the terms and conditions of its authorization under U.S. Army Corps of Engineers Nationwide Permit 12 under which it was constructing this pipeline. Nationwide 12 does not allow the discharge of drilling fluid (a pollutant) from a point source into waters of the United States. 33 U.S.C. 1365 allows an action against who is alleged to be in violation of (A) an effluent limitation or standard under this chapter or (B) an order issued by the Administrator or a State with respect to such a standard or limitation.

4. violation of Sections 301 and Section 402 of the Clean Water Act for discharging pollutants from a point source into waters of the United States without a permit. 33 U.S.C. 1365 allows an action against who is alleged to be in violation of (A) an effluent limitation or standard under this chapter or (B) an order issued by the Administrator or a State with respect to such a standard or limitation.

Remedies sought

The homeowner plaintiffs seek actual damages including, but not limited to, the costs to seek an interim clean water supply to their homes, all costs for cleanup, filtration equipment, remediation of the water under their property, decrease property values, attorneys' fees and costs, and punitive damages for trespass and gross negligence in amount sufficient to deter future recurrences of a similar event on this property or another. Further, all Plaintiffs seek:

- an injunction requiring Kinder Morgan, PHP, and all other responsible parties, to immediately cease operations and implement appropriate steps to prevent the ongoing illegal discharges of fluids, pollutants and contaminants into underground sources of drinking water, waters of the United States, and the environment;

- an injunction requiring Kinder Morgan, PHP, and all other responsible parties, to immediately remove and remediate the fluids, pollutants and contaminants that have been discharged into underground sources of drinking water, waters of the United States, and the environment;

- penalties or fines appropriate under the applicable federal statutes to be paid to the federal government, which range up to $57,317 per violation, per day depending on the statute; and,

- attorneys' fees and costs of court.

As provided in the provisions above, RCRA, SDWA and CWA all allow for citizen suits to be filed for violation of statutory and regulatory prohibitions. These acts all allow actions against the "person" violating the act. The notice period under the SDWA and CWA is 60 days. The notice period under RCRA is 90 days. At the end of those respective periods, Plaintiffs intend to file suit unless appropriate and adequate resolution and safeguards have been reached with Kinder Morgan, PHP, all other responsible parties, and the involved regulatory authorities.

Respectfully,

Jeffery Mundy
Attorney for Plaintiffs

Copies to:

Via U.S. Certified Mail/RRR
Permian Highway Pipeline, LLC
Through its Registered Agent:
Capitol Corporate Services, Inc.
206 E. 9th St., Suite 1300
Austin, Texas 78701

Via U.S. Certified Mail/RRR
Kinder Morgan Texas Pipeline LLC
Through its Registered Agent
Capitol Corporate Services, Inc.
206 E. 9th St., Suite 1300
Austin, Texas 78701

Via U.S. Certified Mail/RRR
Andrew Wheeler, Administrator Environmental Protection Agency
William J. Clinton Bld., Mail Code 1101A
1200 Pennsylvania Avenue, N.W.
Washington, D.C. 20460

Via U.S. Certified Mail/RRR
Ken Paxton, Attorney General
Office of the Attorney General
P.O. Box 12548
Austin, Texas 78711-2548

Via U.S. Certified Mail/RRR
Ken McQueen, Regional Administrator
Environmental Protection Agency, Region 6
1201 Elm Street, Suite 500
Dallas, Texas 75270-210

Via U.S. Certified Mail/RRR
Toby Baker, Executive Director
Office of the Executive Director, MC 109
Texas Commission on Environmental Quality
P.O. Box 13087
Austin, Texas 78711-3087

Via U.S. Certified Mail/RRR
Brent Wade, Deputy Director
Office of Waste, Mail Code 123
Texas Commission on Environmental Quality
P.O. Box 13087
Austin, Texas 78711-3087

Via U.S. Certified Mail/RRR
Dr. Mark T. Esper
Secretary of Defense
1000 Defense Pentagon
Washington, DC 20301-1000

Via U.S. Certified Mail/RRR
Ryan D. McCarthy
Secretary of the Army
101 Army Pentagon
Washington D. C. 20310-0101

Via U.S. Certified Mail/RRR
Col. Kenneth N. Reed
U.S. Army Corp of Engineers
Fort Worth District
819 Taylor Street
Fort Worth, Texas 76102

AMC GEL

AMC

Chemwatch Hazard Alert Code: **3**

Chemwatch: **42071**
Version No: **11.1.1.1**
Safety Data Sheet according to WHS and ADG requirements

Issue Date: **07/07/2017**
Print Date: **02/02/2018**
L.GHS.AUS.EN

SECTION 1 IDENTIFICATION OF THE SUBSTANCE / MIXTURE AND OF THE COMPANY / UNDERTAKING

Product Identifier

Product name	AMC GEL
Other means of identification	Not Available

Relevant identified uses of the substance or mixture and uses advised against

Relevant identified uses	Drilling fluid compound; viscosifier.

Details of the supplier of the safety data sheet

Registered company name	AMC
Address	216 Balcatta Rd Balcatta WA 6021 Australia
Telephone	+61 8 9445 4000
Fax	+61 8 9445 4040
Website	www.amcmud.com
Email	amc@imdexlimited.com

Emergency telephone number

Association / Organisation	**Not Available**
Emergency telephone numbers	1800 039 008 or +61 3 9573 3112,+800 2436 2255 +613 9573 3112
Other emergency telephone numbers	Not Available

SECTION 2 HAZARDS IDENTIFICATION

Classification of the substance or mixture

HAZARDOUS CHEMICAL. NON-DANGEROUS GOODS. According to the WHS Regulations and the ADG Code.

CHEMWATCH HAZARD RATINGS

		Min	Max
Flammability	0		
Toxicity	1		
Body Contact	0		
Reactivity	0		
Chronic	3		

0 = Minimum
1 = Low
2 = Moderate
3 = High
4 = Extreme

Poisons Schedule	Not Applicable
Classification [1]	Carcinogenicity Category 1A, Specific target organ toxicity - repeated exposure Category 1
Legend:	*1. Classified by Chemwatch; 2. Classification drawn from HSIS ; 3. Classification drawn from EC Directive 1272/2008 - Annex VI*

Label elements

Hazard pictogram(s)	
SIGNAL WORD	DANGER

Hazard statement(s)

H350	May cause cancer.
H372	Causes damage to organs through prolonged or repeated exposure.

Precautionary statement(s) Prevention

P201	Obtain special instructions before use.
P260	Do not breathe dust/fume/gas/mist/vapours/spray.

Precautionary statement(s) Response

P308+P313	IF exposed or concerned: Get medical advice/attention.
P314	Get medical advice/attention if you feel unwell.

Precautionary statement(s) Storage

P405	Store locked up.

Precautionary statement(s) Disposal

P501	Dispose of contents/container in accordance with local regulations.

SECTION 3 COMPOSITION / INFORMATION ON INGREDIENTS

Substances

See section below for composition of Mixtures

Mixtures

CAS No	%[weight]	Name
1302-78-9	>94	bentonite
9003-05-8	<0.5	acrylamide homopolymer
497-19-8	<0.5	sodium carbonate
14808-60-7	1-6	silica crystalline - quartz

SECTION 4 FIRST AID MEASURES

Description of first aid measures

Eye Contact	If this product comes in contact with the eyes: ▸ Wash out immediately with fresh running water. ▸ Ensure complete irrigation of the eye by keeping eyelids apart and away from eye and moving the eyelids by occasionally lifting the upper and lower lids. ▸ Seek medical attention without delay; if pain persists or recurs seek medical attention. ▸ Removal of contact lenses after an eye injury should only be undertaken by skilled personnel.
Skin Contact	If skin or hair contact occurs: ▸ Flush skin and hair with running water (and soap if available). ▸ Seek medical attention in event of irritation.
Inhalation	▸ If fumes or combustion products are inhaled remove from contaminated area. ▸ Lay patient down. Keep warm and rested. ▸ Prostheses such as false teeth, which may block airway, should be removed, where possible, prior to initiating first aid procedures. ▸ Apply artificial respiration if not breathing, preferably with a demand valve resuscitator, bag-valve mask device, or pocket mask as trained. Perform CPR if necessary. ▸ Transport to hospital, or doctor. ▸ If dust is inhaled, remove from contaminated area. ▸ Encourage patient to blow nose to ensure clear breathing passages.

	▸ Ask patient to rinse mouth with water but to not drink water. ▸ Seek immediate medical attention.
Ingestion	▸ Immediately give a glass of water. ▸ First aid is not generally required. If in doubt, contact a Poisons Information Centre or a doctor.

Indication of any immediate medical attention and special treatment needed

Treat symptomatically.

SECTION 5 FIREFIGHTING MEASURES

Extinguishing media

- There is no restriction on the type of extinguisher which may be used.
- Use extinguishing media suitable for surrounding area.

Special hazards arising from the substrate or mixture

Fire Incompatibility	None known.

Advice for firefighters

Fire Fighting	▸ Alert Fire Brigade and tell them location and nature of hazard. ▸ Wear breathing apparatus plus protective gloves in the event of a fire.
Fire/Explosion Hazard	▸ Non combustible. ▸ Not considered a significant fire risk, however containers may burn.
HAZCHEM	Not Applicable

SECTION 6 ACCIDENTAL RELEASE MEASURES

Personal precautions, protective equipment and emergency procedures

See section 8

Environmental precautions

See section 12

Methods and material for containment and cleaning up

Minor Spills	▸ Clean up waste regularly and abnormal spills immediately. ▸ Avoid breathing dust and contact with skin and eyes.
Major Spills	▸ Clear area of personnel and move upwind. ▸ Alert Fire Brigade and tell them location and nature of hazard.

Personal Protective Equipment advice is contained in Section 8 of the SDS.

SECTION 7 HANDLING AND STORAGE

Precautions for safe handling

Safe handling	▸ Avoid all personal contact, including inhalation. ▸ Wear protective clothing when risk of exposure occurs.
Other information	▸ Store in original containers. ▸ Keep containers securely sealed.

Conditions for safe storage, including any incompatibilities

Suitable container	▸ Polyethylene or polypropylene container. ▸ Check all containers are clearly labelled and free from leaks.
Storage incompatibility	Silicas: ▸ react with hydrofluoric acid to produce silicon tetrafluoride gas ▸ react with xenon hexafluoride to produce explosive xenon trioxide ▸ reacts exothermically with oxygen difluoride, and explosively with chlorine trifluoride (these halogenated materials are not commonplace industrial materials) and other fluorine-containing compounds ▸ may react with fluorine, chlorates ▸ are incompatible with strong oxidisers, manganese trioxide, chlorine trioxide, strong alkalis, metal oxides, concentrated orthophosphoric acid, vinyl acetate ▸ may react vigorously when heated with alkali carbonates.

SECTION 8 EXPOSURE CONTROLS / PERSONAL PROTECTION

Control parameters

OCCUPATIONAL EXPOSURE LIMITS (OEL)

INGREDIENT DATA

Source	Ingredient	Material name	TWA	STEL	Peak	Notes
Australia Exposure Standards	silica crystalline - quartz	Silica - Crystalline	Not Available	Not Available	Not Available	Not Available
Australia Exposure Standards	silica crystalline - quartz	Quartz (respirable dust)	0.1 mg/m3	Not Available	Not Available	Not Available
Australia Exposure Standards	silica crystalline - quartz	Quartz (respirable dust)	0.1 mg/m3	Not Available	Not Available	Not Available

EMERGENCY LIMITS

Ingredient	Material name	TEEL-1	TEEL-2	TEEL-3
bentonite	Montmorillonite	30 mg/m3	330 mg/m3	2,000 mg/m3
sodium carbonate	Sodium carbonate	7.6 mg/m3	83 mg/m3	500 mg/m3
silica crystalline - quartz	Silica, crystalline-quartz; (Silicon dioxide)	0.075 mg/m3	33 mg/m3	200 mg/m3

Ingredient	Original IDLH	Revised IDLH
bentonite	Not Available	Not Available
acrylamide homopolymer	Not Available	Not Available
sodium carbonate	Not Available	Not Available
silica crystalline - quartz	Not Available	Not Available

MATERIAL DATA

Exposure controls

Appropriate engineering controls	Engineering controls are used to remove a hazard or place a barrier between the worker and the hazard. Well-designed engineering controls can be highly effective in protecting workers and will typically be independent of worker interactions to provide this high level of protection.
Personal protection	
Eye and face protection	▸ Safety glasses with side shields ▸ Chemical goggles. ▸ Contact lenses may pose a special hazard; soft contact lenses may absorb and concentrate irritants.
Skin protection	See Hand protection below
Hands/feet protection	The selection of suitable gloves does not only depend on the material, but also on further marks of quality which vary from manufacturer to manufacturer. Where the chemical is a preparation of several substances, the resistance of the glove material can not be calculated in advance and has therefore to be checked prior to the application. Experience indicates that the following polymers are suitable as glove materials for protection against undissolved, dry solids, where abrasive particles are not present. ▸ polychloroprene.
Body protection	See Other protection below
Other protection	▸ Employees working with confirmed human carcinogens should be provided with, and be required to wear, clean, full body protective clothing (smocks, coveralls, or long-sleeved shirt and pants), shoe covers and gloves prior to entering the regulated area. [AS/NZS ISO 6529:2006 or national equivalent] ▸ Employees engaged in handling operations involving carcinogens should be provided with, and required to wear and use half-face filter-type respirators with filters for dusts, mists and fumes, or air purifying canisters or cartridges. ▸ Prior to each exit from an area containing confirmed human carcinogens, employees should be required to remove and leave protective clothing and equipment at the point of exit and at the last exit of the day, to place used clothing and equipment in impervious containers at the point of exit for purposes of decontamination or disposal. The contents of such impervious containers must be identified with suitable labels. ▸ Overalls. ▸ P.V.C.
Thermal hazards	Not Available

Recommended material(s)

GLOVE SELECTION INDEX

Glove selection is based on a modified presentation of the:
"Forsberg Clothing Performance Index".
The effect(s) of the following substance(s) are taken into account in the ***computer-generated*** selection:
AMC GEL

Material	CPI
NATURAL RUBBER	C
NITRILE	C

* CPI - Chemwatch Performance Index
A: Best Selection
B: Satisfactory; may degrade after 4 hours continuous immersion
C: Poor to Dangerous Choice for other than short term immersion
NOTE: As a series of factors will influence the actual performance of the glove, a final selection must be based on detailed observation. -
* Where the glove is to be used on a short term, casual or infrequent basis, factors such as "feel" or convenience (e.g. disposability), may dictate a choice of gloves which might otherwise be unsuitable following long-term or frequent use. A qualified practitioner should be consulted.

Respiratory protection

Particulate. (AS/NZS 1716 & 1715, EN 143:2000 & 149:001, ANSI Z88 or national equivalent)

If inhalation risk above the TLV exists, wear approved dust respirator.
Use respirators with protection factors appropriate for the exposure level.

- Up to 5 X TLV, use valveless mask type; up to 10 X TLV, use 1/2 mask dust respirator
- Up to 50 X TLV, use full face dust respirator or demand type C air supplied respirator
- Up to 500 X TLV, use powered air-purifying dust respirator or a Type C pressure demand supplied-air respirator
- Over 500 X TLV wear full-face self-contained breathing apparatus with positive pressure mode or a combination respirator with a Type C positive pressure supplied-air full-face respirator and an auxiliary self-contained breathing apparatus operated in pressure demand or other positive pressure mode
- Respirators may be necessary when engineering and administrative controls do not adequately prevent exposures.
- The decision to use respiratory protection should be based on professional judgment that takes into account toxicity information, exposure measurement data, and frequency and likelihood of the worker's exposure - ensure users are not subject to high thermal loads which may result in heat stress or distress due to personal protective equipment (powered, positive flow, full face apparatus may be an option).
- Published occupational exposure limits, where they exist, will assist in determining the adequacy of the selected respiratory protection. These may be government mandated or vendor recommended.
- Certified respirators will be useful for protecting workers from inhalation of particulates when properly selected and fit tested as part of a complete respiratory protection program.
- Use approved positive flow mask if significant quantities of dust becomes airborne.
- Try to avoid creating dust conditions.

SECTION 9 PHYSICAL AND CHEMICAL PROPERTIES

Information on basic physical and chemical properties

Appearance	Bentonite clay (powder) varying in colour from grey to various shades of brown, insoluble in water.

Physical state	Divided Solid	**Relative density (Water = 1)**	Not Available
Odour	Not Available	**Partition coefficient n-octanol / water**	Not Available
Odour threshold	Not Available	**Auto-ignition temperature (°C)**	Not Applicable
pH (as supplied)	Not Applicable	**Decomposition temperature**	Not Available
Melting point / freezing point (°C)	Not Available	**Viscosity (cSt)**	Not Applicable
Initial boiling point and boiling range (°C)	Not Available	**Molecular weight (g/mol)**	Not Applicable
Flash point (°C)	Not Applicable	**Taste**	Not Available
Evaporation rate	Not Applicable	**Explosive properties**	Not Available
Flammability	Not Applicable	**Oxidising properties**	Not Available
Upper Explosive Limit (%)	Not Applicable	**Surface Tension (dyn/cm or mN/m)**	Not Applicable
Lower Explosive Limit (%)	Not Applicable	**Volatile Component (%vol)**	Not Applicable
Vapour pressure (kPa)	Not Applicable	**Gas group**	Not Available
Solubility in water (g/L)	Immiscible	**pH as a solution (1%)**	Not Applicable
Vapour density (Air = 1)	Not Applicable	**VOC g/L**	Not Available

SECTION 10 STABILITY AND REACTIVITY

Reactivity	See section 7
Chemical stability	▸ Unstable in the presence of incompatible materials. ▸ Product is considered stable.
Possibility of hazardous reactions	See section 7
Conditions to avoid	See section 7
Incompatible materials	See section 7
Hazardous decomposition products	See section 5

SECTION 11 TOXICOLOGICAL INFORMATION

Information on toxicological effects

Inhaled	Inhalation of dusts, generated by the material during the course of normal handling, may be damaging to the health of the individual. Effects on lungs are significantly enhanced in the presence of respirable particles. Overexposure to respirable dust may produce wheezing, coughing and breathing difficulties leading to or symptomatic of impaired respiratory function.
Ingestion	The material has NOT been classified by EC Directives or other classification systems as "harmful by ingestion". This is because of the lack of corroborating animal or human evidence.
Skin Contact	The material is not thought to produce adverse health effects or skin irritation following contact (as classified by EC Directives using animal models). Nevertheless, good hygiene practice requires that exposure be kept to a minimum and that suitable gloves be used in an occupational setting. Open cuts, abraded or irritated skin should not be exposed to this material
Eye	Although the material is not thought to be an irritant (as classified by EC Directives), direct contact with the eye may cause transient discomfort characterised by tearing or conjunctival redness (as with windburn). Slight abrasive damage may also result.
Chronic	On the basis of epidemiological data, the material is regarded as carcinogenic to humans. There is sufficient data to establish a causal association between human exposure to the material and the development of cancer. Toxic: danger of serious damage to health by prolonged exposure through inhalation. The health hazards associated with bentonite, kaolin, and common clay, which are commercially important clay products, as well as the related phyllosilicate minerals montmorillonite, kaolinite, and illite, have an extensive literature. Fibrous clay minerals, such as sepiolite, attapulgite, and zeolites, have a separate literature. Chronic symptoms produced by crystalline silicas included decreased vital lung capacity and chest infections. Lengthy exposure may cause silicosis a disabling form of pneumoconiosis which may lead to fibrosis, a scarring of the lining of the air sacs in the lung. Overexposure to respirable dust may cause coughing, wheezing, difficulty in breathing and impaired lung function. Chronic symptoms may include decreased vital lung capacity, chest infections Repeated exposures, in an occupational setting, to high levels of fine- divided dusts may produce a condition known as pneumoconiosis which is the lodgement of any inhaled dusts in the lung irrespective of the effect.

	TOXICITY	IRRITATION
AMC GEL	Not Available	Not Available
bentonite	TOXICITY	IRRITATION
	dermal (rat) LD50: >2000 mg/kg[1]	Not Available
	Inhalation (rat) LC50: >50 mg/l1 h[1]	
	Oral (rat) LD50: >2000 mg/kg[1]	
	Oral (rat) LD50: >5000 mg/kg[1]	
acrylamide homopolymer	TOXICITY	IRRITATION
	Inhalation (rat) LC50: 5.7125 mg/l/30M[2]	Eye: slight
	Oral (rat) LD50: >2000 mg/kg[2]	
sodium carbonate	TOXICITY	IRRITATION
	dermal (rat) LD50: >2000 mg/kg[2]	Eye (rabbit): 100 mg/24h moderate
	Inhalation (guinea pig) LC50: 0.4 mg/l/2h[2]	Eye (rabbit): 100 mg/30s mild
	Oral (rat) LD50: 2800 mg/kg[2]	Eye (rabbit): 50 mg SEVERE

		Skin (rabbit): 500 mg/24h mild
silica crystalline - quartz	TOXICITY Not Available	IRRITATION Not Available
Legend:	*1. Value obtained from Europe ECHA Registered Substances - Acute toxicity 2.* Value obtained from manufacturer's SDS. Unless otherwise specified data extracted from RTECS - Register of Toxic Effect of chemical Substances*	

BENTONITE	Asthma-like symptoms may continue for months or even years after exposure to the material ceases. This may be due to a non-allergenic condition known as reactive airways dysfunction syndrome (RADS) which can occur following exposure to high levels of highly irritating compound. No significant acute toxicological data identified in literature search. for bentonite clays: Bentonite (CAS No. 1302-78-9) consists of a group of clays formed by crystallisation of vitreous volcanic ashes that were deposited in water. The expected acute oral toxicity of bentonite in humans is very low (LD50>15 g/kg).
ACRYLAMIDE HOMOPOLYMER	Sensitisation (guiea pig): 0% (0/20) OECD 406
SILICA CRYSTALLINE - QUARTZ	**WARNING:** For inhalation exposure ONLY: This substance has been classified by the IARC as Group 1: **CARCINOGENIC TO HUMANS** The International Agency for Research on Cancer (IARC) has classified occupational exposures to **respirable** (<5 um) crystalline silica as being carcinogenic to humans . This classification is based on what IARC considered sufficient evidence from epidemiological studies of humans for the carcinogenicity of inhaled silica in the forms of quartz and cristobalite.

Acute Toxicity	⊘	**Carcinogenicity**	✔
Skin Irritation/Corrosion	⊘	**Reproductivity**	⊘
Serious Eye Damage/Irritation	⊘	**STOT - Single Exposure**	⊘
Respiratory or Skin sensitisation	⊘	**STOT - Repeated Exposure**	✔
Mutagenicity	⊘	**Aspiration Hazard**	⊘

Legend: ✘ *– Data available but does not fill the criteria for classification*
✔ *– Data available to make classification*
⊘ *– Data Not Available to make classification*

SECTION 12 ECOLOGICAL INFORMATION

Toxicity

	ENDPOINT	TEST DURATION (HR)	SPECIES	VALUE	SOURCE
AMC GEL	Not Available	Not Available	Not Available	Not Available	Not Available
bentonite	LC50	96	Fish	19000mg/L	4
acrylamide homopolymer	Not Available	Not Available	Not Available	Not Available	Not Available
sodium carbonate	LC50	96	Fish	300mg/L	4
	EC50	48	Crustacea	=176mg/L	1
	EC50	96	Algae or other aquatic plants	242mg/L	4
	NOEC	16	Crustacea	424mg/L	4
silica crystalline - quartz	Not Available	Not Available	Not Available	Not Available	Not Available

Legend:	*Extracted from 1. IUCLID Toxicity Data 2. Europe ECHA Registered Substances - Ecotoxicological Information - Aquatic Toxicity 3. EPIWIN Suite V3.12 (QSAR) - Aquatic Toxicity Data (Estimated) 4. US EPA, Ecotox database - Aquatic Toxicity Data 5. ECETOC Aquatic Hazard Assessment Data 6. NITE (Japan) - Bioconcentration Data 7. METI (Japan) - Bioconcentration Data 8. Vendor Data*

DO NOT discharge into sewer or waterways.
May be harmful to fauna if not disposed of according to Section 13 and legislative requirements. [AMC]

Persistence and degradability

Ingredient	Persistence: Water/Soil	Persistence: Air
acrylamide homopolymer	LOW	LOW
sodium carbonate	LOW	LOW

Bioaccumulative potential

Ingredient	Bioaccumulation
acrylamide homopolymer	LOW (LogKOW = -0.8074)
sodium carbonate	LOW (LogKOW = -0.4605)

Mobility in soil

Ingredient	Mobility
acrylamide homopolymer	LOW (KOC = 10.46)
sodium carbonate	HIGH (KOC = 1)

SECTION 13 DISPOSAL CONSIDERATIONS

Waste treatment methods

Product / Packaging disposal	Legislation addressing waste disposal requirements may differ by country, state and/ or territory. Each user must refer to laws operating in their area. ▸ **DO NOT** allow wash water from cleaning or process equipment to enter drains. ▸ It may be necessary to collect all wash water for treatment before disposal. ▸ Recycle wherever possible or consult manufacturer for recycling options. ▸ Consult State Land Waste Management Authority for disposal.

SECTION 14 TRANSPORT INFORMATION

Labels Required

Marine Pollutant	NO
HAZCHEM	Not Applicable

Land transport (ADG): NOT REGULATED FOR TRANSPORT OF DANGEROUS GOODS

Air transport (ICAO-IATA / DGR): NOT REGULATED FOR TRANSPORT OF DANGEROUS GOODS

Sea transport (IMDG-Code / GGVSee): NOT REGULATED FOR TRANSPORT OF DANGEROUS GOODS

Transport in bulk according to Annex II of MARPOL and the IBC code

Not Applicable

SECTION 15 REGULATORY INFORMATION

Safety, health and environmental regulations / legislation specific for the substance or mixture

BENTONITE(1302-78-9) IS FOUND ON THE FOLLOWING REGULATORY LISTS

Australia Inventory of Chemical Substances (AICS)

ACRYLAMIDE HOMOPOLYMER(9003-05-8) IS FOUND ON THE FOLLOWING REGULATORY LISTS

Australia Inventory of Chemical Substances (AICS)

SODIUM CARBONATE(497-19-8) IS FOUND ON THE FOLLOWING REGULATORY LISTS

Australia Hazardous Substances Information System - Consolidated Lists
Australia Inventory of Chemical Substances (AICS)

SILICA CRYSTALLINE - QUARTZ(14808-60-7) IS FOUND ON THE FOLLOWING REGULATORY LISTS

Australia Exposure Standards
Australia Hazardous Substances Information System - Consolidated Lists
Australia Inventory of Chemical Substances (AICS)
International Agency for Research on Cancer (IARC) - Agents Classified by the IARC Monographs

National Inventory	Status
Australia - AICS	Y
Canada - DSL	Y
Canada - NDSL	N (bentonite; silica crystalline - quartz; acrylamide homopolymer; sodium carbonate)
China - IECSC	N (acrylamide homopolymer)
Europe - EINEC / ELINCS / NLP	N (acrylamide homopolymer)
Japan - ENCS	N (bentonite)
Korea - KECI	Y
New Zealand - NZIoC	Y
Philippines - PICCS	Y
USA - TSCA	Y
Legend:	*Y = All ingredients are on the inventory* *N = Not determined or one or more ingredients are not on the inventory and are not exempt from listing(see specific ingredients in brackets)*

SECTION 16 OTHER INFORMATION

Other information

Ingredients with multiple cas numbers

Name	CAS No
bentonite	1302-78-9, 11004-12-9, 10043-07-9, 115628-71-2, 12198-92-4, 12199-69-8, 135945-01-6, 37320-72-2, 52623-66-2, 850872-77-4, 67479-91-8, 89382-86-5, 90989-60-9, 85049-30-5, 97862-66-3, 84776-12-5, 70131-50-9, 90989-59-6
sodium carbonate	497-19-8, 7542-12-3, 1314087-39-2, 1332-57-6
silica crystalline - quartz	14808-60-7, 122304-48-7, 122304-49-8, 12425-26-2, 1317-79-9, 70594-95-5, 87347-84-0, 308075-07-2

Classification of the preparation and its individual components has drawn on official and authoritative sources as well as independent review by the Chemwatch Classification committee using available literature references.

The SDS is a Hazard Communication tool and should be used to assist in the Risk Assessment. Many factors determine whether the reported Hazards are Risks in the workplace or other settings.

Definitions and abbreviations

PC—TWA: Permissible Concentration-Time Weighted Average
PC—STEL: Permissible Concentration-Short Term Exposure Limit
IARC: International Agency for Research on Cancer
ACGIH: American Conference of Governmental Industrial Hygienists
STEL: Short Term Exposure Limit
TEEL: Temporary Emergency Exposure Limit。
IDLH: Immediately Dangerous to Life or Health Concentrations
OSF: Odour Safety Factor
NOAEL :No Observed Adverse Effect Level
LOAEL: Lowest Observed Adverse Effect Level
TLV: Threshold Limit Value
LOD: Limit Of Detection
OTV: Odour Threshold Value
BCF: BioConcentration Factors
BEI: Biological Exposure Index

Article

Toxicology and Industrial Health

Toxicology and Industrial Health
2020, Vol. 36(1) 11–21

DOI: 10.1177/0748233719900841
journals.sagepub.com/home/tih
SAGE

Effects of bentonite nanoparticles inhalation on lung tissue and blood antioxidant indices in a rat model

Fatemehsadat Masoudi[1], Ali Naghizadeh[2], Mahmoud Zardast[2], Abdullah Gholami[2], Khadijeh Farrokhfall[2], Mohsen Foadoddini[3] and Omid Mehrpour[2]

Abstract

Bentonite is an inorganic clay material that is often easily dispersed as fine particles by air and water circulation, and most people are exposed to different concentrations of bentonite particles. Therefore, the inhaled effects of bentonite nanoparticles (BNPs) were studied in Wistar rats. Seventy-five rats were divided into five groups of 15: four exposure groups (0.1, 0.5, 2, and 10 mg/m^3 of BNPs) and one control group. The rats were exposed for 30, 60, and 90 days to BNPs for 5 days a week (6 h/day) in whole-body inhalation chambers. Blood samples were collected to measure the levels of antioxidant activity of the contents such as total antioxidant capacity (TAC) and malondialdehyde (MDA). X-ray diffraction and scanning electron microscopy were used to identify nanoparticles. The results showed no significant difference in the effect of nanoparticles on levels of TAC and MDA in the studied groups based on the concentrations of nanoparticles. However, the level of MDA increased significantly with extending exposure time; there was a significant increase in the level of MDA content 90 days postexposure compared to 30 days postexposure at concentrations of 0.5, 2, and 10 mg/m^3. Histopathological examination showed that inhalation exposure of rats to BNPs led to different histopathologic responses in the lung tissue, such as inflammatory infiltration, granulomatous inflammation, acute neutrophilic reaction in the early stages, and lung fibrosis. At the lowest concentration, BNPs have low or no toxicity, and inhalation of these nanoparticles at low concentrations does not affect the levels of MDA and TAC content. However, increased concentration and exposure time caused correspondingly greater increases in MDA and more damage to lung tissue.

Keywords

Inhalation exposure, bentonite nanoparticles, lung tissue, malondialdehyde, total antioxidant capacity

Received 14 April 2019; Revised 05 December 2019; Accepted 20 December 2019

Introduction

Nowadays, with the development of science and technology, various materials are used for industrial and consumer products. These materials may have beneficial or harmful effects on human health and living organisms. With the expansion of nanotechnology industrialization, the use of nanoparticles has also increased in modern technologies (Kumar Teli et al., 2010; Whitesides, 2005).

Clay nanoparticles are natural substances that belong to the family of crystalline calcareous

[1]Department of Environment Health Engineering, Student Research Committee, Birjand University of Medical Sciences, Birjand, Iran
[2]Medical Toxicology and Drug Abuse Research Center (MTDRC), Birjand University of Medical Sciences (BUMS), Birjand, Iran
[3]Cardiovascular Research Center, Faculty of Medicine, Birjand University of Medical Sciences (BUMS), Birjand, Iran

Corresponding author:
Ali Naghizadeh, Medical Toxicology and Drug Abuse Research Center, Birjand University of Medical Sciences, Ghaffari Street, P.O. Box 9717853577, Birjand, Iran.
Email: al.naghizadeh@yahoo.com

minerals. Bentonite nanoparticles (BNPs) are also a kind of smectic nanocomposite. They have tetrahedral and octahedral aluminosilicate sheets (Abdou et al., 2013; Banat et al., 2000; Bereket et al., 1997). In recent years, these nanoparticles are used extensively in various industries including electronics, agriculture, food packaging, clothing, pharmaceuticals, cosmetics, sports equipment, medicine, and drug delivery and water purification as hydrocarbon adsorbents (Sirait et al., 2017; Verma et al., 2012). Studies show that millions of tons of bentonite, montmorillonite, and kaolin nanoparticles are used in the ceramic industry (Kryuchkova et al., 2016). The penetration of nanomaterials into various intracellular and extracellular portions, such as the epithelium and mesothelioma cells, is confirmed using electron microscopy (Elsaesser and Howard, 2012). There are certain systems in the body that deal with the damage caused by reactive oxygen species (ROS), which are known as the oxidant defense system. These antioxidant systems are called total antioxidant capacity (TAC). The antioxidant system can prevent the production of ROS, repair the damages caused by radical activity, increase the excretion of damaged molecules, and minimize cell mutations caused by damage from free radicals (Cochrane, 1997; Halliwell and Gutteridge, 1990; Sies, 1993; Uttara et al., 2009; Valko et al., 2006; Wu et al., 2006). Malondialdehyde (MDA) is one of the most important determinants of lipid peroxidation and can be used as a marker to measure oxidative stress levels (Lykkesfeldt, 2007).

Exposure to factors such as environmental pollutants, particles and nanoparticles, drugs, toxins, anesthetic gases, and different rays causes a state called oxidative stress, which can be the basis for more than 100 types of illnesses (Juranek and Bezek, 2005; Malekirad et al., 2005a, 2005b). Nanomaterials, including nanoclay particles such as BNPs, can have unwanted effects on human health (Warheit et al., 2010). Because of the lack of information on the effect of nanoparticles special BNPs on health, this study investigated the effects of BNPs concentration and exposure time on lung damage and levels of antioxidant contents (MDA and TAC) in Wistar rats.

Materials and methods

Bentonite nanoparticles

Pale-yellow BNP powder was purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Table 1 presents the physiochemical properties of the nanoclays used in the present study. The characteristics of BNPs were determined using scanning electron microscopy (SEM) and X-ray diffraction (XRD) analyses. SEM is a powerful magnification instrument for studying the morphological structure of adsorbents, and XRD is a rapid analysis for identifying crystalline material and also for providing information on the dimensions of the nanoparticles (Mahmoud et al., 2016).

Table 1. The physiochemical properties of the nanoparticles used in the present study.

Characterization	Amount
Formula	$H_2Al_2O_6Si$
Appearance (form)	Powder
Bulk density	600–1100 kg/m^3
CAS no.	1302-78-9
Formula weight	180.1 g/mol

Animals and exposure intervals

Seventy-five male Wistar rats (mean body weight: 250 $\pm$ 20 g) were purchased from the Center for Empirical Medicine Research of Birjand, Iran University of Medical Sciences. The animals were approximately 8 weeks old. The rats were housed in polypropylene cages under standard maintenance conditions, including 12-h light and 12-h dark cycle, relative temperature of 22 $\pm$ 2°C, 40–60% humidity, and easy access to water (plastic bottle with screw lid) and complete food (standard food, Javaneh-Khorasan, Mashhad, Iran). The rats were randomly divided into 5 groups of 15 animals (4 exposure groups and 1 control group). The animals were exposed to 0.1, 0.5, 2, and 10 mg/m^3 concentrations of nanoparticles for 6 h/day, 5 days/week for 30, 60, and 90 days in whole-body inhalation chambers. The control group (unexposed) received fresh air during the same exposure period. Five rats from each group were randomly euthanized 30 days postexposure, five animals were euthanized 60 days postexposure, and five animals were euthanized 90 days postexposure (30, 60, and 90 days exposure, $n = 5$). This research project was approved at 2018 by the Ethical Committee of Birjand University of Medical Sciences with ethical coded: Ir.bums. REC. 1397.7.

Inhalation exposure

Seamless plastic dishes measuring 36 × 40 × 55 cm^3 were used to construct chambers equipped with three control valves to expose the rats to BNPs powder.

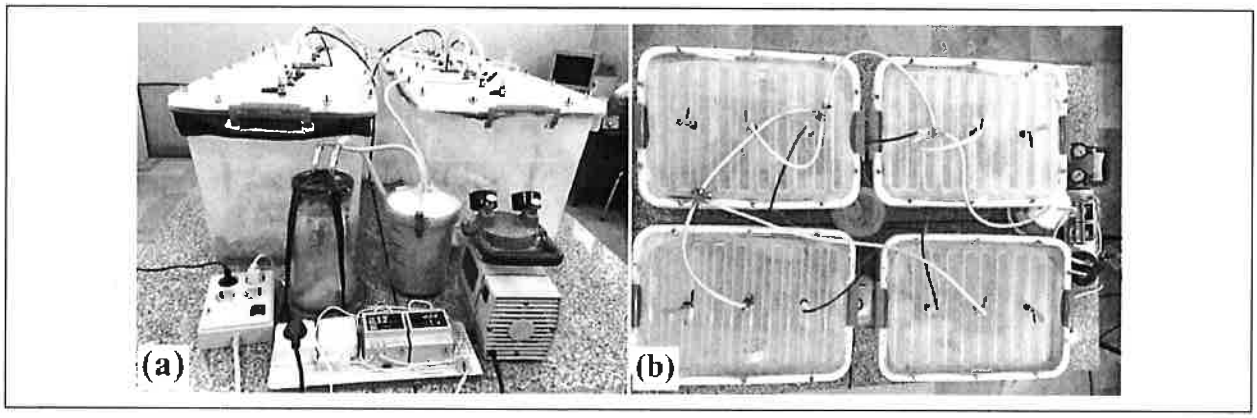

Figure 1. Exposure chambers. (a) Side view of air pump connection, dish containing nanoparticles, equalization, and control circuit. (b) Connection of the series of exposure chambers.

The dust flow was brought into the chambers by one of the valves, and by another valve, clean air entered the chambers. Another valve was used for sampling. The dust concentrations were made by an air pump (model GM-0.50 Diaphragm Vacuum Pump, Holliston, Massachusetts, USA) inside a dish containing BNPs powder. In order to equalize the dust flow, it was entered into an equalization dish and then led through the interface hoses into the chambers. Dust flow measurements inside the chambers were performed by an aerosol generation system (TSI Model 8520-dust track, flow rate = 0.001–100 mg/m^3, Shoreview, Minnesota, USA). The in vivo inhalation toxicity inhalation in this study was conducted according to the OECD testing guideline TG 413 (OECD, 2018). The concentration of the dust inside the chambers was adjusted with control valves. Figure 1 shows the chambers for nanoparticles exposure of the rats.

Oxidative stress assay

At this stage, 5 cc of heart blood samples were taken, and after centrifugation with the speed of 15,000 r/min for 20 min (Kubota, Model KN-70, Kyoto, Japan), their plasma was collected in microtube by sampler and stored in −20°C. TAC was measured using the ferric reduction of antioxidant power (FRAP) method. The basis of FRAP measurement is the reduction of ferric ions by reducing antioxidant activity in the presence of a representative of tripyridyl-s-triazine (TPTZ) resulting in a Fe^{2+}-TPTZ blue complex, which was measured in mmol/l by a spectrophotometer at the wavelength of 593 nm. Also, the amount of MDA was determined by adding 200 µl/ml of 67% thiobarbituric acid and 600 µl/ml of 1% phosphoric acid to 100 µl of sample. The sample was heated in a boiling water bath (90–100°C) for 45 min. Then, 800 µl of 1-butanol was added to samples, and after centrifugation (5000 r/min for 20 min), the absorbance was measured in µmol/l by an EPOCH (USA) spectrophotometer at the wavelength of 532 nm. Also, 1,1,3,3 tetraethoxypropane at different concentrations was used as MDA standard (Benzie and Strain, 1996; Kei, 1978; Uchiyama and Mihara, 1978).

Lung tissue assay

After the exposure periods, the rats were anesthetized using diethyl ether and euthanized humanely, and the lungs of the rats were carefully removed from the body. The lungs were fixed in 10% buffered formalin. Then, paraffin parasagital sections (5-µm thickness) were prepared and stained by hematoxylin–eosin for histological analysis. Finally, the samples were examined under a light microscope (40, 100, and 400×) (Olympus, Model CX31, Philippines).

Statistical analysis

SPSS software (version 22, IBM, Chicago, USA) was used to perform the statistical calculations. The data and results of the experiments were expressed as mean ± SD. Analysis of variance and multiple Duncan tests were used for statistical evaluation. *P*-value less than 0.05 was considered statistically significant.

Results

Characteristics of BNPs

SEM analysis. Figure 2 displays the morphological structure of BNPs used in this study as shown by SEM. As can be observed in the figure, BNPs are

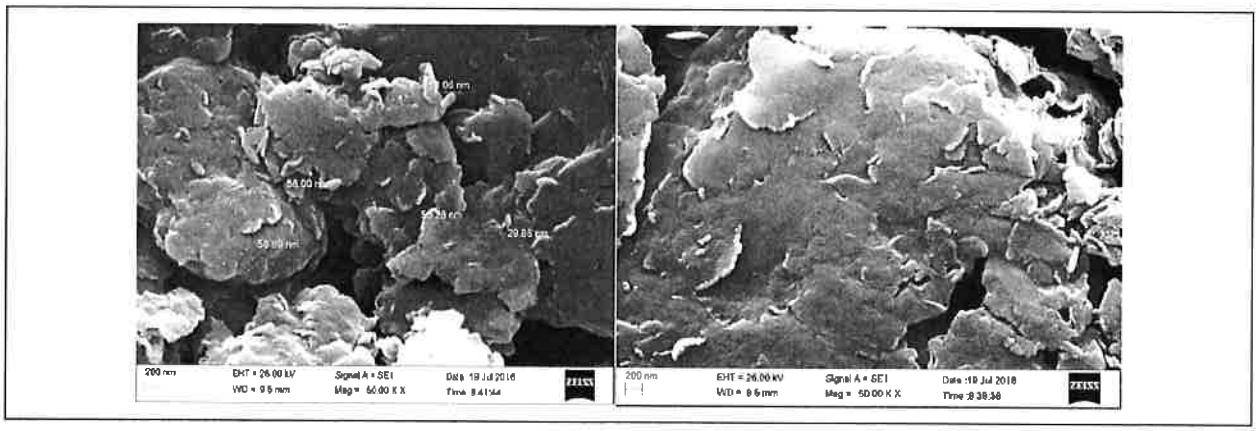

Figure 2. Scanning electron microscopic images of bentonite nanoparticles.

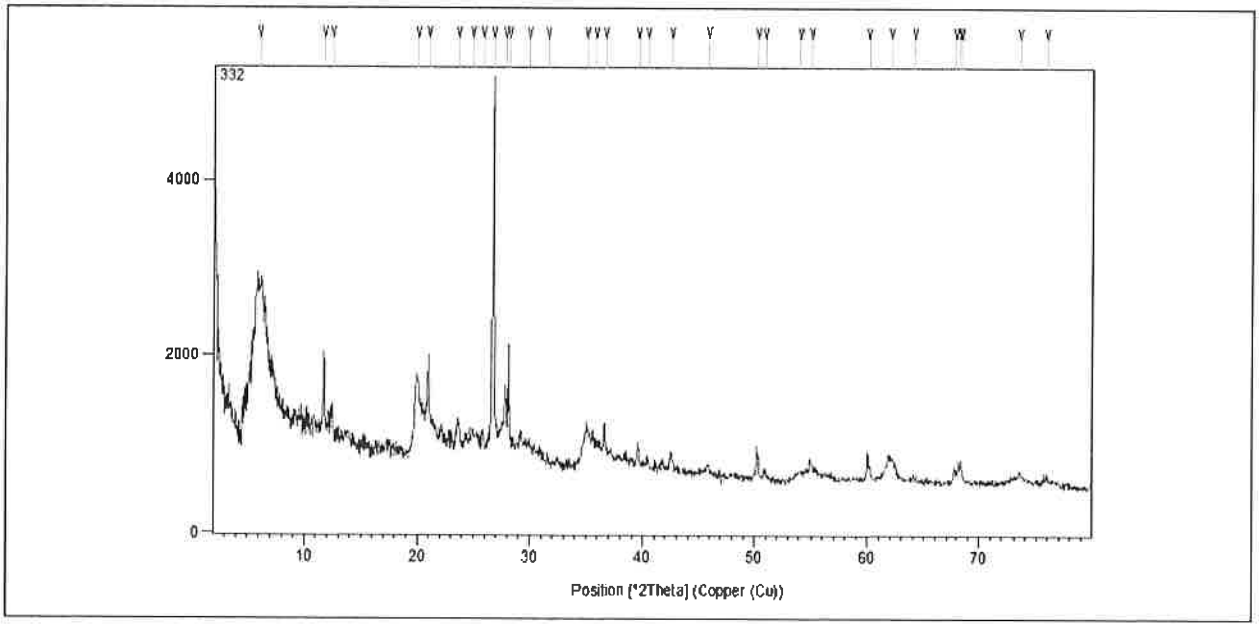

Figure 3. X-Ray powder diffraction pattern of the bentonite nanoparticles.

nanosheets that are arranged in layers. The thickness of each of the sheets is about 29–70 nm.

XRD analysis. The XRD analysis of BNPs is shown in Figure 3. As it is clear from the figure, the medium and sharp peaks of BNPs are $2\theta = 6.05°$ and $2\theta = 26.71°$, respectively. This is in accordance with the nanostructures of particles. To calculate the size of nanoparticles based on XRD spectrum graph data, the Debye–Scherrer equation (equation (1)) was used (Bhatia et al., 2017).

$$D = \frac{k\lambda}{\beta \cos \theta} \quad (1)$$

where D denotes diameter of particles, β shows peak width of the diffraction peak profile at half-maximum height resulting from small crystallite size (full width at half maximum), θ is angle of diffraction, and λ is equal to 1.54 Å.

The size of the nanoparticles was calculated to be 77.43 nm according to the calculations of the Scherrer equation.

Oxidative stress assay

The levels of TAC content 30, 60, and 90 days post-exposure are shown in Figure 4. The results of the effect of BNPs on the level of TAC content of male rats at 0.1, 0.5, 2, and 10 mg/m^3 concentrations of

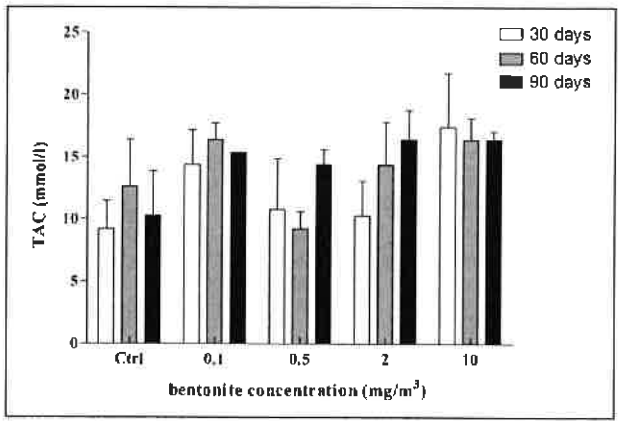

Figure 4. Levels of TAC content after inhalation exposure to bentonite nanoparticles. Values are mean ± SD of five animals/group. TAC: total antioxidant capacity; BNPs: bentonite nanoparticles.

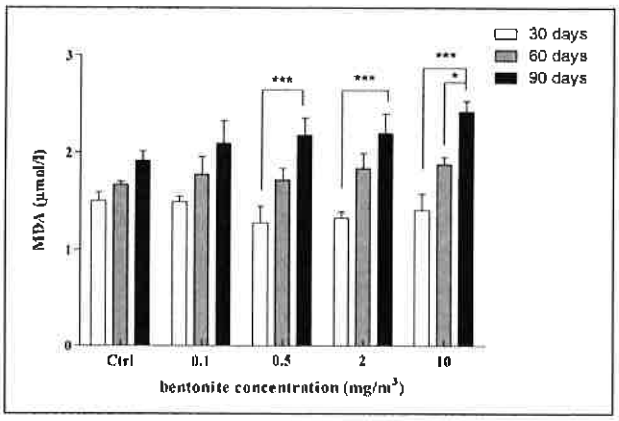

Figure 5. Levels of MDA content after inhalation exposure of bentonite nanoparticles. Values are mean ± SD of five animals/group. *$p < 0.05$; ***$p < 0.001$, versus same exposure concentration groups. MDA: malondialdehyde; BNPs: bentonite nanoparticles.

nanoparticles 30, 60, and 90 days postexposure showed that the level of TAC content had increased. However, no significant differences were observed in any exposure concentrations and groups ($p > 0.05$). Also, there was no significant difference in the levels of TAC content after the three postexposure times between the five study groups.

Comparison of the levels of MDA content between the inhaled BNPs exposure groups at concentrations of 0.1, 0.5, 2, and 10 mg/m^3 and the control group 30, 60, and 90 days postexposure is shown in Figure 5. There was no significant difference in the levels of MDA content between the five groups after 30, 60, and 90 days of inhalation exposure ($p > 0.05$). With regard to the level of MDA content based on the exposure times, there was a significant increase in the level of MDA content 90 days postexposure compared to 30 days postexposure at the concentrations of 0.5, 2, and 10 mg/m^3 ($p < 0.001$). Also, there was a significant difference in the level of MDA content in the group exposed to 10 mg/m^3 concentration of BNPs between 60 days and 90 days postexposure ($p = 0.002$).

Lung tissue assay

The histological effects of BNPs at the concentrations of 0.1, 0.5, 2, and 10 mg/m^3 inhaled for 30, 60, and 90 days on lung tissue of rats were investigated. The histopathological examinations showed that the inhalation exposure of rats to BNPs caused histopathological alterations in the lung tissues of the five groups such as inflammatory lesions, macrophage accumulation, acute neutrophilic reaction, granulomatous inflammation, and pulmonary fibrosis. Exposure in different groups increased the rate and severity of histopathological changes compared to the control group.

Figure 6 shows the accumulation of macrophages over different exposure times at the highest studied concentration of BNPs. Relatively low accumulation of macrophages was observed in the alveolar duct within 30 days postexposure (see Figure 6(b)). Sixty days postexposure, these changes were characterized by the accumulation of abundant macrophages inside and in the alveolar duct, and after 90 days of exposure, these changes were more pronounced (see Figure 6(c) and (d)).

Figure 7 presents granuloma formation in the 10 mg/m^3 concentration of BNPs at different exposure times. Granulomatous inflammation intensified with increased exposure time, such that 90 days postexposure, granulomatous inflammation was accompanied by an increase in alveolar septal thickening due to inflammation and fibrosis (see Figure 7(d)). Figure 8 exhibits the changes in pulmonary fibrosis in the 10 mg/m^3 concentration of bentonite nanoparticles over different times of exposure. Fibrosis was accompanied by an increase in the thickness of the alveolar septal wall, low lymphocytes infiltrations and macrophages in the alveolar duct within 30 days (see Figure 8(b)). Sixty days postexposure, moderate fibrosis in the alveolar duct was noted, which was more severe in some places (see Figure 8(c)). Ninety days postexposure, severe fibrosis in the alveolar duct, increased thickness of the alveolar septal wall, and the presence

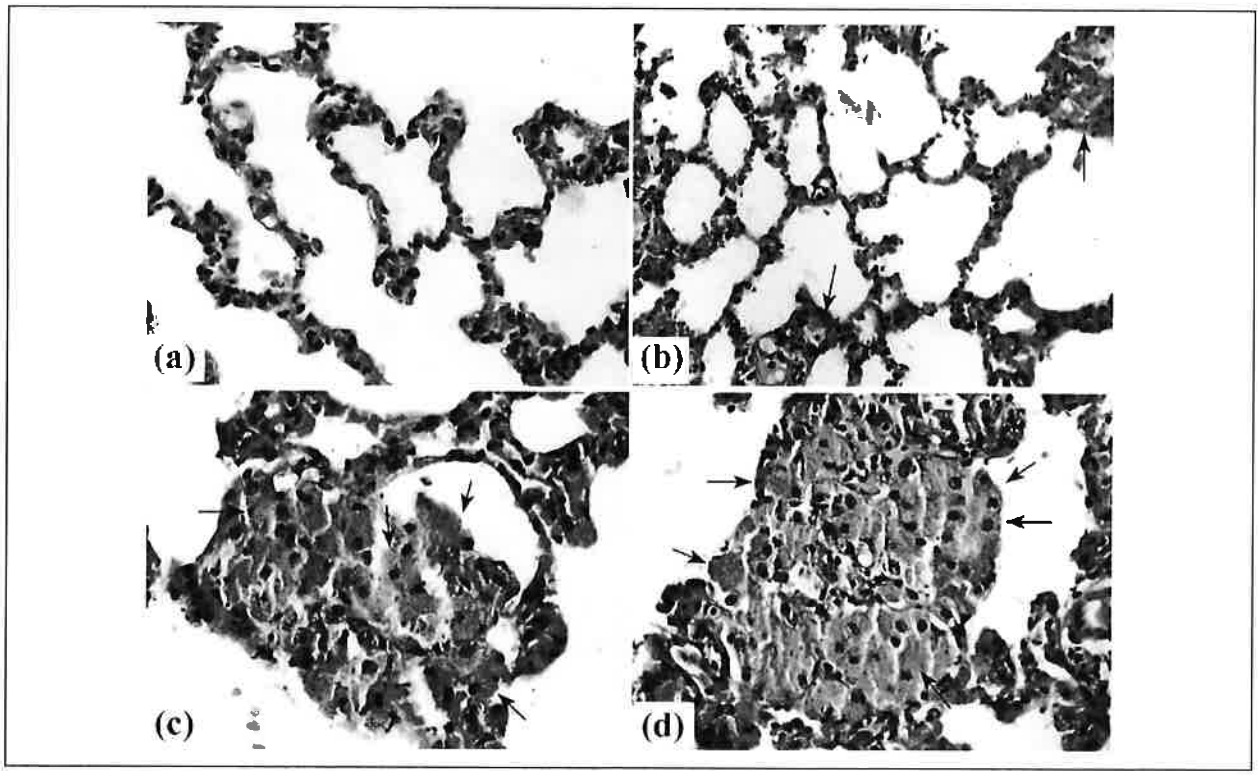

Figure 6. Macrophage accumulation (arrows). (a) Control group, (b) 30 days postexposure, the presence of a small number of macrophages in the inside and the alveolar duct, (c) 60 days postexposure, the accumulation of macrophages was high and mainly in the alveolar region and a small amount in the alveolar duct, and (d) 90 days postexposure, many macrophages in the alveolar region and the alveolar duct (10 mg/m^3 of BNPs, H&E staining, ×400). BNPs: bentonite nanoparticles; H&E: hematoxylin–eosin.

of a small necrotic area were observed (see Figure 8(d)). Acute neutrophilic reactions were observed among the exposed groups 30 days postexposure. However, 60 and 90 days after exposure, acute neutrophilic reaction was not observed among the exposed groups. Figure 9 shows acute neutrophilic reaction in the 30-day exposure period at the highest concentration of BNPs. Neutrophils were a sign of reaction and were not observed in the samples obtained 60 and 90 days postexposure. Also, granulomatous inflammation in the lymph node of the lung tissue was detectable with foreign body giant cells (Figure 10).

Discussion

Because of the widespread (adverse) effect of nanoparticles especially long-term exposure on health, the effect of BNPs concentration and exposure time on levels of antioxidant contents (TAC and MDA) and lung damage in Wistar rats was investigated. According to Figures 2 and 3 and also the Scherrer equation, the BNPs are nanosheets of less than 100-nm thickness. Also two peaks in the XRD analysis ($2\theta = 6.05°$ and $2\theta = 26.71°$) are related to montmorillonite aluminum silicate and quartz peaks, respectively (Shahwan et al., 2010; Vieira et al., 2010).

According to Figure 4, BNPs did not affect the TAC level of rats at all concentrations and exposure times. However, MDA levels increased with extending exposure time, which was significant. There was a significant increase in the level of MDA content 90 days postexposure compared to 30 days postexposure at concentrations of 0.5, 2, and 10 mg/m^3 and a significant difference in the exposure group at 10 mg/m^3 concentration of BNPs between 60 days and 90 days postexposure indicating increased oxidative stress. In a study conducted by Kryuchkova et al. (2016) on the effect of clay nanoparticles on *Paramecium caudatum*, the results showed that 10 mg/ml of clay nanoparticles had no significant effect on levels of MDA during 24 h (increase of 3–6%). They also reported that these nanoparticles had low or no toxicity at the considered concentration and did not change the

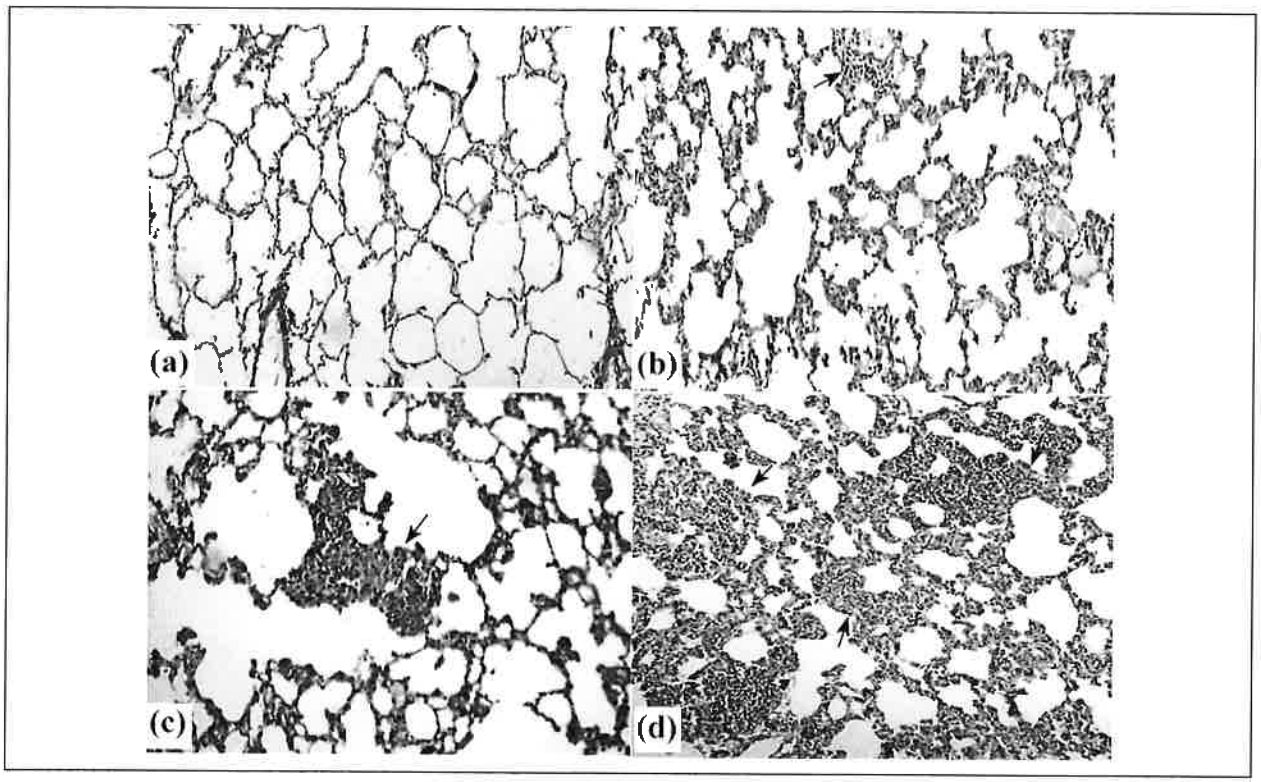

Figure 7. Granuloma formation (arrows). (a) Control group, (b) 30 days postexposure, primary granuloma reaction, (c) 60 days postexposure, granulomatous inflammation in the central region and scattered macrophage accumulation, and (d) 90 days postexposure, there was granulomatous inflammation, along with an increase in the thickness of the alveolar septal wall and fibrosis (10 mg/m^3 of BNPs, H&E staining, × 100). BNPs: bentonite nanoparticles; H&E: hematoxylin–eosin.

oxidative stress level (Kryuchkova et al., 2016). Regarding a study conducted by Maisanaba et al. (2014) on the effect of clay mineral nanoparticles on antioxidant enzymes of male Wistar rats with 40 mg/kg/day oral exposure, it was reported that clay nanoparticles had no effect on the levels of MDA in the liver and kidneys. As a result, these nanoparticles did not play a role in changing levels of oxidative stress (Maisanaba et al., 2014). In another similar study by Shi et al. (2006), it was found that oral exposure to montmorillonite nanocomposite had no effect on the levels of superoxide dismutase, glutathione peroxidase, and MDA parameters of the liver and blood serum of broiler chickens (Shi et al., 2006). In a study performed by Zhang et al. (2010), the toxicity and oxidative stress caused by two types of bentonite particles in human B lymphoblast cells at concentrations of 30, 60, 120, and 240 μg/ml were investigated in vitro for 6 h. The results showed that increased concentration of bentonites particle enhanced cytotoxic effects and oxidative stress in human B lymphoblastic cells (Zhang et al., 2010).

Some of studies have attributed the effect of nanoparticles on living organism cells to characteristics such as the diameter, shape, size, and nature of nanoparticles (Carretero et al., 2013; Moudgil and Roberts, 2001). Many minerals can be beneficial or toxic depending on the dose or exposure time (Gomes and Silva, 2007). Also, studies on BNPs confirm that increased dosage and exposure times of nanoparticles may have negative effects on health (Carretero et al., 2013). In another study conducted by Yuwen et al. (2013), the inhalation effects of BNPs on genetic damage and lipid peroxidation were investigated. The results showed that over exposure to these mineral substances could lead to detectable genetic damages and lipid peroxidation, which may be affected by exposure to various concentrations of organic BNPs (Huang et al., 2013). However, other studies on the effect of bentonite showed that these particles are not toxic to humans, and this mineral has been approved as a food additive in different countries such as Australia (Maisanaba et al., 2015).

In the present study, after necropsy and study of histopathologic changes in the lung tissue of the rats

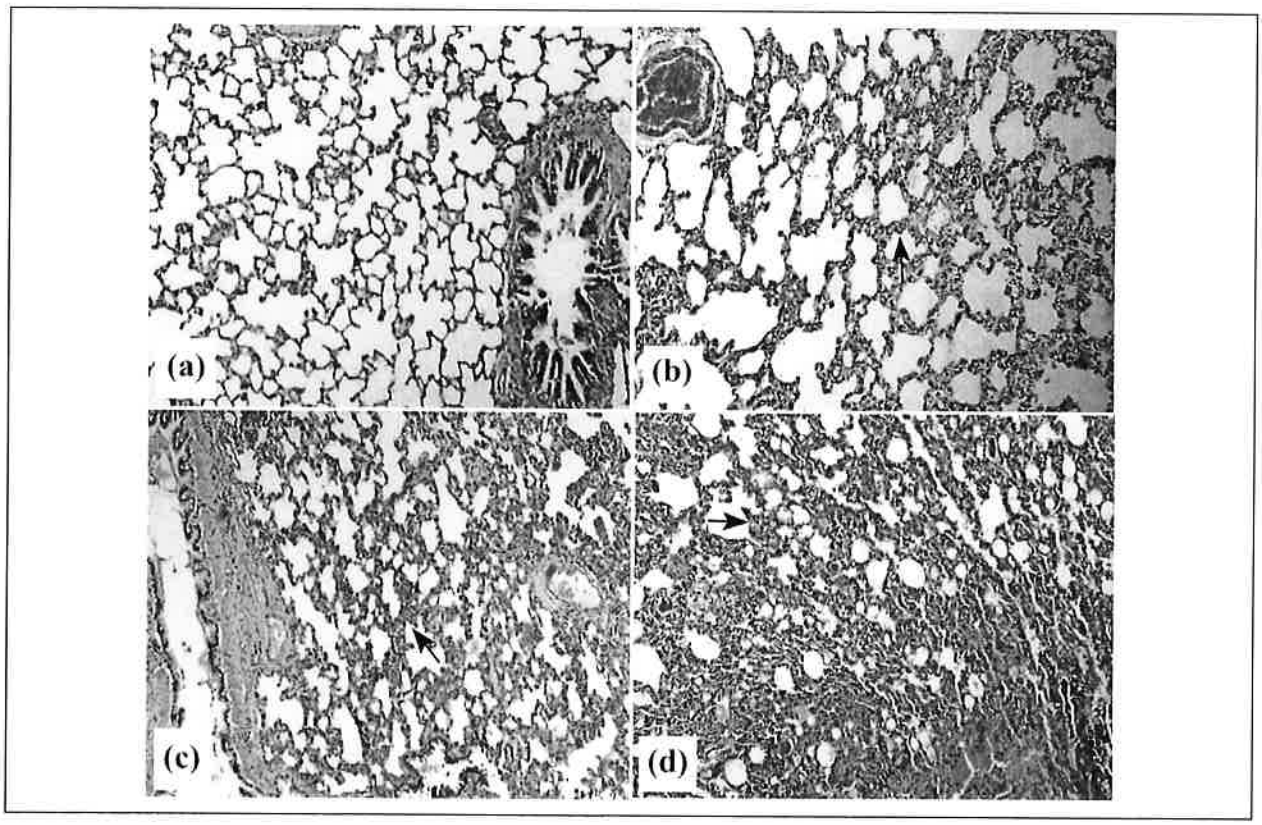

Figure 8. Pulmonary fibrosis (arrows). (a) Control group, (b) 30 days postexposure, fibrosis and increased thickness in the alveolar duct and low infiltration of lymphocytes and macrophages in the alveolar duct, (c) 60 days postexposure, the presence of moderate fibrosis in the alveolar duct which is more severe in some places, and (d) 90 days postexposure, severe fibrosis in the alveolar duct, increased thickening in the alveolar duct, and the presence of a small necrotic cavity (10 mg/m^3 of BNPs, H&E staining, ×40). BNPs: bentonite nanoparticles; H&E: hematoxylin–eosin.

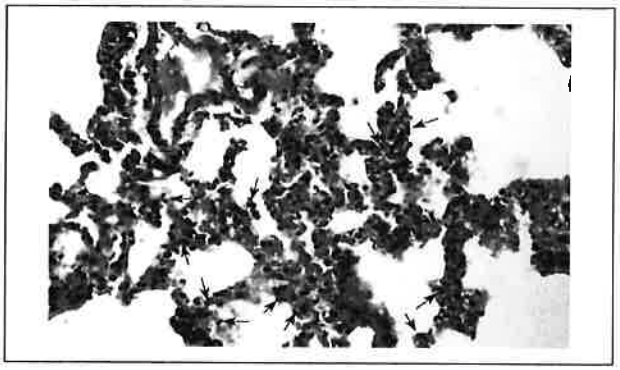

Figure 9. Acute neutrophilic reaction. Neutrophil infiltration (arrows) is observed in the alveolar duct (10 mg/m^3 of BNPs, 30 days postexposure, H&E staining, ×100). BNPs: bentonite nanoparticles; H&E: hematoxylin–eosin.

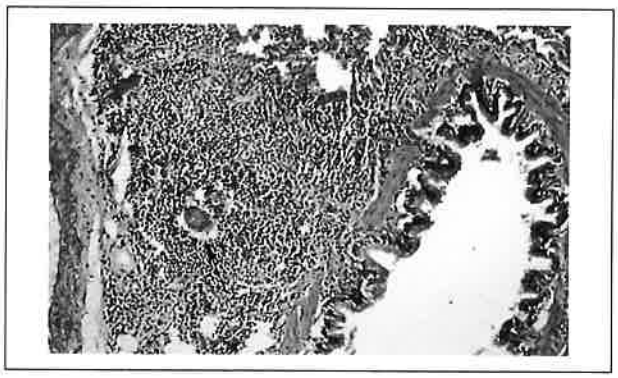

Figure 10. Granulomatous inflammation in the lymph node with foreign body giant cells (10 mg/m^3 of BNPs, 90 days postexposure, H&E staining, ×100). BNPs: bentonite nanoparticles; H&E: hematoxylin–eosin.

exposed to the inhalation of BNPs, it was shown that these nanoparticles caused different alternations. Also, the results of this study and its histological observations confirm that BNPs caused less tissue damage at low concentrations, while high concentrations of BNPs had a greater effect on lung tissue; in other words, the effect of BNPs on lung tissue was dose-dependent. The results also indicated that lung

damage in short-term exposure was mild, and with extending the duration of exposure, the severity and extend of damage to the lung increased. The histopathological examinations in this study showed that the inhalation exposure of rats to BNPs caused histopathological alterations such as inflammatory lesions, macrophage accumulation, acute neutrophilic reaction, granulomatous inflammation, and pulmonary fibrosis in the lung tissues. The adverse health effects of nanoparticles are increasingly dependent on their specific characteristics, including the composition of particles, electrostatic charge, and the reactivity associated with biological systems (Oberdörster et al., 2005; Powers et al., 2006).

Regarding to the results of the present study, pulmonary pathological changes depend on the concentration and duration of exposure to clay nanoparticles. Long-term exposure to BNPs can lead to pulmonary inflammation, fibrosis, pneumonia, and other diseases of the lung (Elmore, 2003; Maisanaba et al., 2015; Maxim et al., 2016). Other studies have also shown that inhalation of these nanoparticles results in pulmonary fibrosis, which can be transformed into lung cancer or mesothelioma (Carretero et al., 2013). These pathologic changes are similar to those of other mineral aluminosilicate nanoparticles, including montmorillonite, sepiolite, talc, and kaolin (Gibbs, 1990; Gibbs and Pooley, 1994). In a study conducted by Bolton et al. regarding the effects of inorganic silicate nanoparticles, dust exposure at a concentration of 10 mg/m^3 for 12 months on lung tissue showed that all groups exposed to nanoparticles had macrophages which containing dust throughout the alveolar regions of the lung are associated with an increase in the thickness of the alveolar septal wall and interstitial fibrosis (Bolton et al., 1986). The results of the study performed by Navin et al. on effects of clay nanoparticle toxicity on in vitro human epithelial A549 cells showed a small but significant level of cell cytotoxicity in A549 cells exposed to 25 µg/ml of BNPs, cloisite, and hydrophilic bentonite. Also, at higher concentrations, cell growth ability depended on exposure time and concentration with a maximum loss of cellular concentration at the highest concentration (250 µg/ml) (Verma et al., 2012). In a study by Gibbs and Pooley on the pathological examination of lung tissue, long-term exposure to montmorillonite mineral nanoparticles led to pneumoconiosis and interstitial collections of dust-laden macrophages with slight fibrosis (Gibbs and Pooley, 1994). In a study conducted by Warheit et al., histopathological evaluation of rat lung tissue showed that exposure to sepiolite nanoclays led to inflammation and lung damage after 24 h of exposure. Nanoparticles of sepiolite after 3 months of exposure also caused multinucleated giant cell accumulation, increased alveolar duct thickness, and increased lung changes (Warheit et al., 2010). Also, Gibbs et al. (1992) investigated the effect of talc mineral particles on lung tissue. They characterized the pathological characteristics of individuals exposed to the inhalation of talc and reported the accumulation of macrophages containing mineral nanoparticles, various degrees of fibrosis, along with giant cells (Gibbs et al., 1992). Research on the lung toxicity of kaolin inorganic nanoparticles in rats has shown that these nanoscale minerals can be fatal at very high concentrations (Zhu and Njuguna, 2014). Also, inhalation of kaolin nanoparticles by workers caused pulmonary fibrosis (Churg and Wiggs, 1985; Dougherty et al., 1985; Johnson et al., 1986). Animal studies assessing the potential toxicity of BNPs are relatively limited. However, the existing studies show that exposure to BNPs at high concentrations and prolonged exposures can directly or indirectly have adverse effects on the lungs (Maxim et al., 2016).

Conclusion

Inhalation of BNPs does not affect the levels of MDA and TAC at low concentrations. However, during long-term exposure and at higher concentrations, BNPs increase the level of MDA as a result of increased oxidative stress. Histological results showed that the absorption of BNPs causes different alternations including lung inflammation, macrophage accumulation, granuloma formation, acute neutrophilic reaction, and pulmonary fibrosis in exposed groups compared to the control group. By increasing the concentration and exposure time of BNPs, more severe damages to lung tissues can be observed. Given the toxicity of these nanomaterials to health, further studies are suggested to predict these nanoparticles on other tissues and body fluids at other concentrations and exposure times.

Authors' note

This article was extracted from an MSc thesis in environmental health engineering and was approved by the Birjand University of Medical Sciences coded 455584 (ethical code: IR.BUMS.1394.373).

Acknowledgement

The authors would like to acknowledge the generous support of the university.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship and/or publication of this article: The authors received financial support from Birjand University of Medical Sciences, Birjand, Iran for the research.

ORCID iD

Ali Naghizadeh https://orcid.org/0000-0002-3015-2609

References

Abdou M, Al-Sabagh A and Dardir M (2013) Evaluation of Egyptian bentonite and nano-bentonite as drilling mud. *Egyptian Journal of Petroleum* 22(1): 53–59.

Banat F, Al-Bashir B, Al-Asheh S, et al. (2000) Adsorption of phenol by bentonite. *Environmental Pollution* 107(3): 391–398.

Benzie IF and Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry* 239(1): 70–76.

Bereket G, Arog AZ and Özel MZ (1997) Removal of Pb (II), Cd (II), Cu (II), and Zn (II) from aqueous solutions by adsorption on bentonite. *Journal of Colloïd and Interface Science* 187(2): 338–343.

Bhatia M, Rajulapati SB, Sonawane S, et al. (2017) Synthesis and implication of novel poly acrylic acid)/nanosorbent embedded hydrogel composite for lead ion removal. *Scientific Reports* 7(1): 16413.

Bolton R, Addison J, Davis J, et al. (1986) Effects of the inhalation of dusts from calcium silicate insulation materials in laboratory rats. *Environmental Research* 39(1): 26–43.

Carretero M, Gomes C and Tateo F (2013) Clays, drugs, and human health. *Developments in Clay Science* 5: 711–764.

Churg A and Wiggs B (1985) Mineral particles, mineral fibers, and lung cancer. *Environmental Research* 37(2): 364–372.

Cochrane C (1997) Cellular injury by antioxidants. *The American Journal of Medicine* 91(3): 23–30.

Dougherty SH, Flegel VD, Nelson RD, et al. (1985) Effects of soil infection potentiating factors on neutrophils in vitro. *The American Journal of Surgery* 150(3): 306–311.

Elmore AR (2003) Final report on the safety assessment of aluminum silicate, calcium silicate, magnesium aluminum silicate, magnesium silicate, magnesium trisilicate, sodium magnesium silicate, zirconium silicate, attapulgite, bentonite, Fuller's earth, hectorite, kaolin, lithium magnesium silicate, lithium magnesium sodium silicate, montmorillonite, pyrophyllite, and zeolite. *International Journal of Toxicology* 22(1): 37–102.

Elsaesser A and Howard CV (2012) Toxicology of nanoparticles. *Advanced Drug Delivery Reviews* 64(2): 129–137.

Gibbs A (1990) Human pathology of kaolin and mica Pneumoconioses. *Health Related Effects of Phyllosilicates* 21: 217–226.

Gibbs A and Pooley F (1994) Fuller's earth (montmorillonite) pneumoconiosis. *Occupational and Environmental Medicine* 51(9): 644–646.

Gibbs A, Pooley F, Griffiths D, et al. (1992) Talc pneumoconiosis: a pathologic and mineralogic study. *Human Pathology* 23(12): 1344–1354.

Gomes C and Silva J (2007) Minerals and clay minerals in medical geology. *Applied Clay Science* 36(1-3): 4–21.

Halliwell B and Gutteridge JM (1990) The antioxidants of human extracellular fluids. *Archives of Biochemistry and Biophysics* 280(1): 1–8.

Huang Y, Zhang M, Zou H, et al. (2013) Genetic damage and lipid peroxidation in workers occupationally exposed to organic bentonite particles. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 751(1): 40–44.

Johnson NF, Haslam PL, Dewar A, et al. (1986) Identification of inorganic dust particles in bronchoalveolar lavage macrophages by energy dispersive x-ray microanalysis. *Archives of Environmental Health: An International Journal* 41(3): 133–144.

Juranek I and Bezek S (2005) Controversy of free radical hypothesis: reactive oxygen species-cause or consequence of tissue injury. *General Physiology and Biophysics* 24(3): 263.

Kei S (1978) Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. *Clinica Chimica Acta* 90(1): 37–43.

Kryuchkova M, Danilushkina A, Lvov Y, et al. (2016) Evaluation of toxicity of nanoclays and graphene oxide in vivo: a *Paramecium caudatum* study. *Environmental Science: Nano* 3(2): 442–452.

Kumar Teli M, Mutalik S and Rajanikant G (2010) Nanotechnology and nanomedicine: going small means aiming big. *Current Pharmaceutical Design* 16(16): 1882–1892.

Lykkesfeldt J (2007) Malondialdehyde as biomarker of oxidative damage to lipids caused by smoking. *Clinica Chimica Acta* 380(1-2): 50–58.

Mahmoud ME, Nabil GM, El-Mallah NM, et al. (2016) Kinetics, isotherm, and thermodynamic studies of the adsorption of reactive red 195 A dye from water by modified Switchgrass Biochar adsorbent. *Journal of Industrial and Engineering Chemistry* 37: 156–167.

Maisanaba S, Pichardo S, Puerto M, et al. (2015) Toxicological evaluation of clay minerals and derived nanocomposites: a review. *Environmental Research* 138: 233–254.

Maisanaba S, Puerto M, Gutiérrez-Praena D, et al. (2014) In vivo evaluation of activities and expression of antioxidant enzymes in Wistar rats exposed for 90 days to a modified clay. *Journal of Toxicology and Environmental Health, Part A* 77(8): 456–466.

Malekirad AA, Ranjbar A, Rahzani K, et al. (2005a) Oxidative stress in operating room personnel: occupational exposure to anesthetic gases. *Human & Experimental Toxicology* 24(11): 597–601.

Malekirad AA, Ranjbar A, Rahzani K, et al. (2005b) Oxidative stress in radiology staff. *Environmental Toxicology and Pharmacology* 20(1): 215–218.

Maxim LD, Niebo R and McConnell EE (2016) Bentonite toxicology and epidemiology–a review. *Inhalation Toxicology* 28(13): 591–617.

Moudgil B and Roberts S (2001) Designing a strategies for safety evaluation of nanomaterials. Part nano-interface in a microfluidic chip to probe living VI. Characterization of nanoscale particles for cells: challenges and perspectives. *Virtual* 1(1): 6419–6424.

Oberdörster G, Maynard A, Donaldson K, et al. (2005) Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. *Particle and Fibre Toxicology* 2(1): 1–35.

OECD (2018) *Test No. 413: Subchronic Inhalation Toxicity: 90-day Study, OECD Guidelines for the Testing of Chemicals, Section 4*. Paris: OECD Publishing. DOI: 10.1787/9789264070806-en.

Powers KW, Brown SC, Krishna VB, et al. (2006) Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. *Toxicological Sciences* 90(2): 296–303.

Shahwan T, Üzüm Ç, Eroğlu AE, et al. (2010) Synthesis and characterization of bentonite/iron nanoparticles and their application as adsorbent of cobalt ions. *Applied Clay Science* 47(3-4): 257–262.

Shi Y, Xu Z, Feng J, et al. (2006) Efficacy of modified montmorillonite nanocomposite to reduce the toxicity of aflatoxin in broiler chicks. *Animal Feed Science and Technology* 129(1-2): 138–148.

Sies II (1993) Strategies of antioxidant defense. *European Journal of Biochemistry* 215(2): 213–219.

Sirait M, Bukit N and Siregar N (2017) Preparation and characterization of natural bentonite in to nanoparticles by co-precipitation method. In: *AIP Conference Proceedings* (eds Tahir D, Halide H, Surungan T, et al), Vol. 1801, Makassar, Indonesia, 19–21 September 2016.

Uchiyama M and Mihara M (1978) Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Analytical Biochemistry* 86(1): 271–278.

Uttara B, Singh AV, Zamboni P, et al. (2009) Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Current Neuropharmacology* 7(1): 65–74.

Valko M, Rhodes C, Moncol J, et al. (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions* 160(1): 1–40.

Verma NK, Moore E, Blau W, et al. (2012) Cytotoxicity evaluation of nanoclays in human epithelial cell line A549 using high content screening and real-time impedance analysis. *Journal of Nanoparticle Research* 14(9): 1137.

Vieira MGA, Neto AA, Gimenes M, et al. (2010) Sorption kinetics and equilibrium for the removal of nickel ions from aqueous phase on calcined Bofe bentonite clay. *Journal of Hazardous Materials* 177(1-3): 362–371.

Warheit DB, Sayes CM, Frame SR, et al. (2010) Pulmonary exposures to Sepiolite nanoclay particulates in rats: resolution following multinucleate giant cell formation. *Toxicology Letters* 192(3): 286–293.

Whitesides GM (2005) Nanoscience, nanotechnology, and chemistry. *Small* 1(2): 172–179.

Wu BJ, Kathir K, Witting PK, et al. (2006) Antioxidants protect from atherosclerosis by a heme oxygenase-1 pathway that is independent of free radical scavenging. *Journal of Experimental Medicine* 203(4): 1117–1127.

Yuwen H, Meibian Z, Hua Z, et al. (2013) Genetic damage and lipid peroxidation in workers occupationally exposed to organic bentonite particles. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 751(1): 40–44.

Zhang M, Lu Y, Li X, et al. (2010) Studying the cytotoxicity and oxidative stress induced by two kinds of bentonite particles on human B lymphoblast cells in vitro. *Chemico-Biological Interactions* 183(3): 390–396.

Zhu H and Njuguna J (2014) Nanolayered silicates/clay minerals: uses and effects on health. In: Njuguna J, Zhu H and Pielichowski K (eds) *Health and Environmental Safety of Nanomaterials*. Cambridge: Woodhead publishing, pp. 133–146.