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RESEARCH ARTICLE

Evaluation of Possible Ameliorative Role of Robinetin to Counteract Polystyrene Microplastics Instigated Renal Toxicity in Rats

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A B S T R A C T

This investigation was planned to determine the ameliorative potential of robinetin (RN) to counteract the adverse effects of polystyrene microplastics (PSMPs) on renal tissues. Twenty-four albino rats (*Rattus norvegicus*) were distributed into four groups i.e., control, PSMPs (0.01 mg/kg), PSMPs (0.01 mg/kg) + RN (50 mg/kg) and RN (50 mg/kg) only treated group. The activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GSR) were reduced while the levels of reactive oxygen species (ROS) and malondialdehyde (MDA) were escalated following the PSMPs intoxication. Moreover, PSMPs exposure increased the levels of urea, creatinine, KIM-1 and NGAL while downregulating the levels of creatinine clearance. Furthermore, PSMPs markedly escalated the levels of NF-κB, IL-6, IL-1β, TNF-α and COX-2 activity. Besides, the levels of Caspase-3, Bax and Caspase-9 were elevated while levels of Bcl-2 were reduced after PSMPs intoxication. Additionally, PSMPs administration instigated various histopathological anomalies in renal tissues. Nonetheless, RN supplementation considerably restored aforementioned dysregulations instigated by PSMPs due to its antioxidative, anti-apoptotic and anti-inflammatory potential.

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INTRODUCTION

Plastic products have become the dire need of our daily life. Plastics products are extensively used in numerous industries including health care, electronics, medicine, clothing, and agriculture due to their exceptional properties such as high durability, flexibility, low cost, and light weight (Surendran *et al.,* 2023). The immense manufacturing as well as inadequate disposal resulted in massive accumulation of plastic in our environment (Surendran *et al.,* 2023). The degradation of plastic polymers results in a massive production of microplastics (MPs) (Leistenschneider *et al.,* 2023). Numerous investigations reaffirmed that MPs are preset in honey, salt, tea bags, drinking water and seafood (Vandermeersch *et al.,* 2015; Buteler *et al.,* 2023).

Polystyrene microplastics (PSMPs) are widely used as MPs owing to their high tensile strength, low cost, transparency and durability (Alberto and Tsochatzis, 2023). The excessive accumulation of PSMPs instigates organ damage including heart, liver, spleen, and kidney (Zhang *et al.,* 2024). Wang *et al.* (2021) elucidated that PSMPs intoxication induces inflammation and autophagy in renal tissues of mice. Furthermore, PSMPs exposure instigates apoptosis as well as disrupt the normal architecture of renal tissues in human (Wang *et al.,* 2021). It is reported that PSMPs administration escalates the levels of inflammatory and apoptotic markers in renal tissues (Meng *et al.,* 2022). Furthermore, PSMPs contamination impairs the normal renal function via inhibiting antioxidant enzymes activities (Chen *et al.,* 2022).

Natural compounds have demonstrated a wide range of therapeutic potential against various disorders (Chen *et al.,* 2018). Robinetin (RN) is a plant-based flavonoid which exhibits numerous biological abilities i.e., anti-cancerous, antioxidative and anti-mutagenic (Schmitt-Schillig *et al.,* 2005; Tarahovsky *et al.,* 2008). The present study was designed to assess the efficacy of RN to avert PSMPs provoked renal damages in rats.

MATERIALS AND METHODS

Chemicals: Both PSMPs (CAS No. 9003-53-6, Purity: HPLC ≥98.0%) and RN (CAS No. 490-31-3, Purity: HPLC ≥95.0%) were procured from Merck, Germany.

Animals: Twenty-four rats (*Rattus norvegicus*) weighing (200±20g) were kept at the animal house of University of Agriculture, Faisalabad (UAF). The rats were acclimatized to standard laboratory conditions (22-25ºC temperature, 55-55% humidity and 12 hours of light and dark period). Standard rodent nutrition and tap water were provided. During the trial, ethical guidelines of "European Union of Animal Care and Experimentation" were strictly observed, and study was approved by the ethical committee of UAF.

Experimental layout: Twenty-four rats were divided into four groups. The group 1st was designated as the control group. The group 2nd was referred to as PSMPs (0.01 mg/kg) treated group. The group 3rd was subjected to a concurrent dose of PSMPs (0.01 mg/kg) + RN (50 mg/kg). Group 4th was exposed to RN (50 mg/kg) only. After 28 days of trial, the rats were anesthetized by using Ketamine (60mg/kg) and Xylazine (6mg/kg) and decapitated. Heparin containing syringes were used to obtain samples while the kidneys were excised from the body. The left kidney was immersed in formalin solution (10%) for histology while the right kidney was packed in zipper bag and stored at -20℃. Centrifugation was carried out (12000 rpm) at 4ºC for 15 minutes and supernatant was used for biochemical assessment.

Biochemical assessment: The methodology described by Aebi (1974) was followed to quantify CAT activity. The protocol of Kakkar *et al*. (1984) was used to determine SOD activity. The activity of GPx and GSR were evaluated by using the approaches described by Rotruck *et al.* (1973) and Carlberg and Mannervik (1975) respectively. The contents of GSH were assessed by following the methodology delineated by Jollow *et al.* (1974). The ROS and MDA levels were quantified by following the strategy elucidated by Hayashi *et al*. (2007) and Ohkawa (1979).

Assessment of renal function profile: The levels of urea (Cat No. ab83362), creatinine (Cat No. ab65340) and creatinine clearance (Cat No. ab65340), were quantified by using standard ELISA kits (R & D System China Co. Ltd., Changning, China). The levels of NGAL and KIM-1 were evaluated by using NGAL Quantikine ELISA Kits & KIM-1 Quantikine ELISA Kits (R and D Systems company Ltd. Changning, China) respectively. The protocol was executed strictly following the instructions of the manufacturers.

Assessment of inflammatory profile: TNF-α (CSB-E07379r), NF-κB (CSB-E13148r), IL-6 (CSB-E04640r), IL-1β (CSB-E08055r) levels and COX-2 (CSB-E13399r) activity were determined by using standard ELISA kits (R & D, Minnesota, USA). The assessment was carried out according to the instructions of the manufacturer.

Assessment of apoptotic indices: Bcl-2 (CSB-E08854r), Bax (CSB-EL002573RA), Caspase-9 (CSB-E08863r) and Caspase-3 (CSB-E08857r) levels were quantified by using standard ELISA kits (Cusabio Technology Llc, Houston, TX, USA). The protocol was carried out by following the instructions of the manufacturer.

Histopathological analysis: The renal tissues were fixed in solution of 10% formalin and subjected to dehydration by using ascending grades of ethanol. Then the tissues were fixed in paraffin wax and subsequently trimmed into thick pieces (thickness =4-5µm) by using rotatory microtome. The samples were stained by hematoxylin/eosin. The histopathological alterations were observed by using a light microscope at 400X, and microphotographs were captured to observe the histopathological damage.

Statistical analysis: The values were represented as Mean ± SE. One-way (ANOVA) followed by Tukey's test was applied to compare different groups by using Minitab software. The level of significance was set at $P<0.05$.

RESULTS

Impacts of RN on oxidative profile: PSMPs exposure notably ($P<0.05$) decreased antioxidant enzymes activities while upregulating ROS and MDA concentrations. Nevertheless, supplementation of PSMPs + RN considerably ($P<0.05$) escalated the activities of abovementioned antioxidant enzymes whereas reduced ROS and MDA levels. Nonetheless, no significant differences were observed among the values of RN alone and control group (Table 1).

Impacts of RN on renal biomarkers: PSMPs intoxication substantially ($P<0.05$) escalated the levels of NGAL, KIM-1, urea as well as creatinine while decreasing the levels of creatinine clearance. However, PSMPs + RN treatment markedly ($P<0.05$) reduced NGAL, KIM-1, urea as well as creatinine while increasing the levels of creatinine clearance. Furthermore, RN alone treated group showed no discrepancies when compared with the control group (Table 2).

Impacts of RN on inflammatory profile: PSMPs exposure remarkably ($P<0.05$) increased NF-κB, IL-6, TNF-α, and IL-1β levels and COX-2 activity. Nonetheless, co-treatment with PSMPs + RN substantially ($P<0.05$) reduced the abovementioned inflammatory cytokines levels. Nevertheless, RN alone treated group demonstrated insignificant differences when compared with the control group (Table 3).

Impacts of RN on apoptotic markers: PSMPs exposure markedly ($P<0.05$) augmented the levels of Bax, Caspase-9 and Caspase-3 while downregulating the levels of Bcl-2. However, co-administration of PSMPs + RN substantially ($P<0.05$) reduced the levels of Bax, Caspase-3 and Caspase-9 while upregulating the levels of Bcl-2. Furthermore, insignificant differences were observed between RN alone and control group (Table 4).

Impacts of RN on renal histology: PSMPs intoxication significantly ($P<0.05$) increased histological damage such as tabular dilation, vacuolation, glomerulitis, thickening of

Table 1: Effect of RN on antioxidant profile and oxidative stress markers

Parameters	Groups			
	Control	PSMPs	PSMPs + RN	RN
CAT (U/mg protein)	13.51 ± 0.91^{a}	6.36 ± 0.51^{c}	10.52 ± 1.00^{b}	13.82 ± 1.31^{a}
SOD (U/mg protein)	10.35 ± 1.06^{a}	4.34 ± 0.21^{c}	8.00 ± 0.77^{b}	10.58 ± 1.19^{a}
GSR (nM NADPH oxidized/min/mg tissue)	8.32 ± 0.58^{a}	2.98 ± 0.79^{c}	5.52 ± 0.30^{b}	8.41 ± 0.88^{a}
GPx (U/mg protein)	29.15 ± 2.22^{a}	10.59 ± 1.41^{c}	21.82 ± 1.28^{b}	30.12 ± 2.63^{a}
GSH (U/mg protein)	23.12 ± 1.70^{a}	8.26 ± 1.10^{c}	16.85 ± 1.30^{b}	23.84 ± 1.94^{a}
GST (U/mg protein)	35.99 ± 3.10^{a}	15.29 ± 1.83^{c}	27.38 ± 1.85^{b}	36.92 ± 4.13^{a}
MDA (nmol/g)	0.78 ± 0.15^{c}	2.76 ± 0.21^{a}	1.29 ± 0.24^{b}	0.71 ± 0.14^{c}
ROS (nmol/g)	1.13 ± 0.38^{bc}	6.43 ± 0.45^{a}	2.08 ± 0.11^{b}	1.06 ± 0.42^{c}

Note: Values with different superscripts within rows are significantly different from other groups.

Table 2: Effects of RN on renal function markers

Parameters	Groups			
	Control	PSMPs	PSMPs + RN	RN
Urea (mg/dl)	12.53 ± 1.32^{c}	54.63 ± 3.13^{a}	19.49 ± 2.17^{b}	11.79 ± 0.74^{c}
Creatinine (mg/dl)	1.33 ± 0.28^{c}	6.76 ± 0.44^{a}	2.69 ± 0.31^{b}	1.27 ± 0.40^{c}
Creatinine Clearance (ml/min)	2.03 ± 0.18^{a}	0.57 ± 0.20^{c}	1.45 ± 0.09^{b}	2.07 ± 0.12^{a}
KIM-1 (mg/ml)	0.26 ± 0.21^{c}	3.29 ± 0.34^{a}	1.15 ± 0.17^{b}	0.22 ± 0.20^{c}
NGAL (ng/day)	0.81 ± 0.12^{c}	4.36 ± 0.27^{a}	1.77 ± 0.20^{b}	0.76 ± 0.14^{c}

Note: Values with different superscripts within rows are significantly different from other groups.

Table 3: Effects of RN on inflammatory profile

Parameters	Groups			
	Control	PSMPs	PSMPs + RN	RN
NF-kB (ng/g tissue)	17.29 ± 1.01^{c}	73.45 ± 2.50^{a}	27.10 ± 2.23^{b}	16.88 ± 1.20^{c}
TNFα (ng/g tissue)	6.67 ± 0.49^{c}	33.98 ± 2.67^{a}	18.85 ± 1.66^{b}	6.63 ± 0.51^{c}
IL-1ß (ng/g tissue)	32.08 ± 2.9^{b}	83.95 ± 6.61^{a}	38.93 ± 3.56^{b}	31.16 ± 2.64^{b}
IL-6 (ng/g tissue)	9.68 ± 1.62^{c}	35.41 ± 2.40^{a}	19.66 ± 1.84^{b}	9.11 ± 1.95^{c}
COX-2 (ng/g tissue)	17.69 ± 1.78^{c}	68.21 ± 3.06^{a}	27.32 ± 2.24^{b}	17.45 ± 2.02^{c}

Note: Values with different superscripts within rows are significantly different from other groups.

Table 4: Effects of RN on apoptotic indices

Parameters	Groups			
	Control	PSMPs	PSMPs + RN	RN
Bax (pg/mL)	1.56 ± 0.23^{c}	7.85 ± 0.43^{a}	3.05 ± 0.25^{b}	1.51 ± 0.22^{c}
Caspase-3 (pg/mL)	1.08 ± 0.21^{b}	15.29 ± 2.58^{a}	2.67 ± 0.32^{b}	1.01 ± 0.27^{b}
Caspase-9 (pg/mL)	2.71 ± 0.09^{b}	16.22 ± 1.86^{a}	3.55 ± 0.21^{b}	2.65 ± 0.18^{b}
Bcl-2 (ng/mL)	19.26 ± 2.45^{a}	6.10 ± 0.73^{c}	12.44 ± 0.97^{b}	20.21 ± 2.36^{a}

Note: Values with different superscripts within rows are significantly different from other groups.

epithelial walls and necrosis. However, PSMPs + RN treatment markedly ($P<0.05$) revoked aforementioned histological dysregulations. Furthermore, RN alone and the control group demonstrated normal renal histology (Fig. 1).

DISCUSSION

Due to immense production and low disposal rates, plastic pollution adversely affects the health of humans and the ecosystem (Borrelle *et al.,* 2020). The anthropogenic activities worsen the plastic pollution in terrestrial ecosystem as compared to oceans (Horton *et al.,* 2017). PSMPs exhibited the ability to break the blood-brain barriers and accumulate in body organs (Wang *et al.,* 2021). RN is extracted from *R. pseudacacia* which exhibited various pharmacological as well as biological properties such as anti-mutagenic, anti-cancerous, anti-oxidative and anti- leishmanial (Chaudhuri *et al.,* 2010). Due to adverse impacts of PSMPs on kidney, this research was conducted to ascertain the palliative efficacy of RN as countermeasure against renal damages.

Our findings revealed that PSMPs intoxication reduced antioxidant enzymes activities while escalating the levels of ROS and MDA. Antioxidant enzymes exert a substantial protective role to counteract the excessive burden of reactive species. These enzymes break down the free radicals to harmless molecules such as oxygen and alcohol (Ighodaro and Akinloye, 2018). CAT is an endogenous antioxidant enzyme which is responsible for the degradation of hydrogen peroxide into O_2 and H_2O (Chelikani *et al.,* 2004). GPx degrades the lipid peroxidases into their alcohols (Góth *et al.,* 2004). The excessive generation of ROS attacks plasma membrane lipids culminating in the formation of free radicals as well as aldehydes such as MDA (Sies and Jones, 2020). It is reported that oxidative stress is the major culprit behind cellular toxicity (Li *et al.,* 2023). Oxidative stress disrupts the equilibrium of endogenous antioxidant enzymes which leads to the onset of various diseases (Garcia-Caparros *et al.,* 2021). Almundarij and Tharwat (2023) elucidated that OS markers can be used as prognostic indicators during renal injury. The current investigation exhibited that RN supplementation remarkably maintained the oxidative balance by improving antioxidant profile while suppressing the levels of abovementioned oxidants. Kumar *et al.* (2013) elucidated that flavonoids contain multiple hydroxyl functional groups which are responsible for their antioxidant ability.

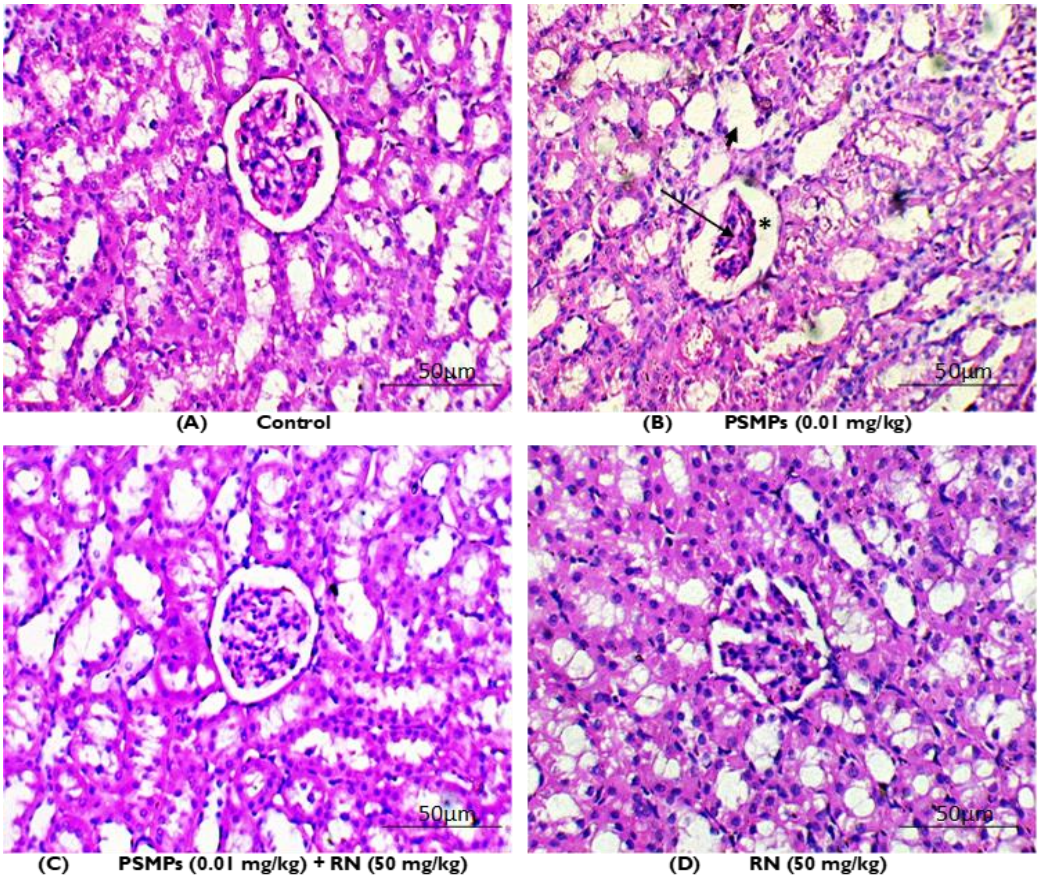

Fig. 1: Renal histology is normal in control group. B) PSMPs group exhibiting shrinkage of glomerulus (arrow), elevated bowman's space (*) and tubular necrosis (arrowhead); C) PSMPs + RN group is illustrating restoration of renal parenchyma (arrowhead) and glomerulus (arrow) D) RN group is demonstrating normal glomerulus (arrow) and renal parenchyma (H&E; 400X).

In the present investigation, PSMPs exposure escalated the levels of creatinine, urea, NGAL and KIM-1 while downregulating the levels of creatinine clearance. Chronic kidney diseases are diagnosed by a progressive reduction in renal functions such as regulation of body fluid as well as excretion of toxic material (Venkatapathy *et al.*, 2014). Urea and creatinine are by-products which need to be excreted from the body through the renal system. Elevation in the levels of these biomarkers indicate serious renal impairment (Kamal, 2014). NGAL and KIM-1 are the most sensitive biomarkers of renal impairment which are released into the bloodstream due to various renal damages such as acute kidney injury, renal tabular damage, and renal ischemia (Tsigou *et al.*, 2013). However, NR treatment depicted a remarkable reduction in creatinine, urea, KIM-1 and NGAL levels while increasing the levels of creatinine clearances.

PSMPs administration escalated the COX-2 activity as well as TNF-α, NF-κB, IL-6, and IL-1β levels. Renal inflammation is an intricate process which occurs due to imbalance of parenchymal cells, renal macrophages, lymphocytes and neutrophils. The activation of these cells mediates the activation of NF-κB and its associated inflammatory cytokines (Andrade-Oliveira *et al.*, 2019). The excessive oxidation of biomolecules causes a substantial elevation in the concentrations of pro-inflammatory cytokines in kidneys (Podkowińska and Formanowicz, 2020). Nonetheless, RN supplementation considerably reduced aforementioned inflammatory cytokines levels which might be owing to its ability to counteract inflammation.

PSMPs exposure considerably upregulated Caspase-9, Caspase-3 and Bax levels whereas reduced Bcl-2 levels. Apoptosis is a natural phenomenon of cell death to maintain a biological system (Priante *et al.*, 2019). The Caspase-3 activation is a crucial phase in the process of apoptosis thereby consider as a sensitive biomarker to ascertain cellular apoptosis (Yu *et al.*, 2014). Bcl-2 and Bax are biomarkers of apoptosis and any change in their ratio predicts the shift of apoptotic balance in the cell (Maione *et al.*, 2015). Nevertheless, RN supplementation remarkably decreased pro-apoptotic profile while upregulating the anti-apoptotic marker.

PSMPs intoxication instigates tabular necrosis, desquamation of epithelial cells, glomerulus inflammation and vacuolization of renal tissues. Our outcomes are further supported by an investigation executed by Wang *et al.* (2021) which demonstrate that PSMPs intoxication enhance formation of ROS and prompted LP via reducing antioxidants balance in kidneys and resulting in histological damages. Furthermore, RN administration significantly restored aforementioned histopathological damages instigated by PSMPs.

Conclusions: PSMPs intoxication reduced antioxidant enzymes activities while upregulating the levels of oxidative stress. Furthermore, PSMPs exposure increased the levels of kidney function markers. Moreover, the levels of pro-apoptotic and inflammatory markers were escalated following the PSMPs exposure. PSMPs intoxication disrupted renal histology. However, RN protected the renal tissues via regulating antioxidant, apoptotic, inflammatory, and histological impairments induced by PSMPs.

Authors contribution: MUI, AN and KAG designed the study. MFH and NE carried out the experiment. UA helped

in statistical analysis. MUI, MFH and KAG wrote and proofread the final version of manuscript. All the authors approved the final submission of the manuscript.

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