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EFFECT OF MAGNESIUM CHLORIDE AND SODIUM FLUORIDE ON VARIOUS HYDROXYPROLINE FRACTIONS IN RAT KIDNEYS

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ABSTRACT

Magnesium chloride ($MgCl_2$) has been reported to protect against sodium fluoride (NaF) induced toxicity. This study was undertaken to study the effect of $MgCl_2$ on NaF induced alteration in rat kidney hydroxyproline fractions and collagen. Four groups of rats were studied (each consisting of 4-6 rats) (i) normal rats: (ii) rats injected with $MgCl_2$: (iii) rats injected with NaF: (iv) rats injected with $MgCl_2$ followed by NaF. Results show that $MgCl_2$ and NaF treatment alone and together caused a significant ($p < 0.05$) decrease in kidney protein, free, peptide–bound, protein-bound, total hydroxyproline and soluble collagen hydroxyproline. Administration of $MgCl_2$ before NaF did not restore the altered parameters to normal levels. However administration of $MgCl_2$ before NaF restored insoluble collagen hydroxyproline which was altered by NaF to near normal levels. Though $MgCl_2$ has been reported to be protective against the toxic effect of NaF, it has no significant effect on NaF induced changes in kidney hydroxyproline/collagen except insoluble collagen Hyp.

Keywords: Sodium fluoride; hydroxyproline; collagen; magnesium chloride.

INTRODUCTION

Fluorine occurs in environment in combination with other elements as a fluoride compound (Manna *et al.*, 2007). Human beings are exposed to fluoride through food (Stannard *et al.*, 1991; Dabek and McKenzie, 1995), drinking water (Zhao, 1996) and inhalation (Gritsan *et al.*, 1995). Frequent absorption of the fluoride causes tooth decay (Neurath, 2005), damage of kidneys (Lantz *et al.*, 1987), bones (Bezerra de Menezes *et al.*, 2003), nerves (Shivarajashankara *et al.*, 2002) and muscles (Cicek *et al.*, 2005). The adverse toxic effects of fluoride arise due to a) enzyme inhibition, b) collagen break down, c) gastric damage and d) disruption of the immune system (Ahmad *et al.*, 2000). Magnesium is a mineral that is involved in over 300 reactions in the body. It is important for every organ in the body, particularly the heart, muscles, and kidneys. It also contributes to the composition of teeth and bones. Most importantly, it activates enzymes, contributes to energy production, and helps regulate the levels of other minerals in the body (Saris *et al.*, 2000).

The kidneys excrete waste products of metabolism and play an important role in maintaining the homeostasis by regulating the body water and solute balance. In addition to the excretory function, the kidneys also have an endocrine function producing hormones like renin, erythropoietin etc. The most commonly used medium for studying fluoride toxicity is urine. Acute exposure to high doses of fluoride damages renal tissue and causes renal dysfunction (Zabulyte *et al.*, 2007). In our previous studies (Al-Omireeni *et al.*, 2009) we have shown that different doses of NaF have profound effect on various hydroxyproline (Hyp) fractions in rat kidneys. Hyp is a component amino acid of collagen and has been used as an index of collagen turnover (Reddy and Enwemeka., 1996). Magnesium chloride ($MgCl_2$) has been reported to exert a protective effect on sodium fluoride (NaF) induced mortality in rats (Luoma *et al.*, 1984). The present study was carried out to study the effect of $MgCl_2$ on NaF induced changes in Hyp fractions and collagen content in rat kidneys.

MATERIALS AND METHODS

Chemicals

Chloramine-T, p-dimethylaminobenzaldeyde (Ehrlich's reagent), L-hydroxyproline, sodium acetate, citric acid, perchloric acid, n-propanol, sodium hydroxide, and acetic acid were purchased from Sigma Chemical Company, St Louis, MO, USA. Double distilled water was used throughout the study.

Animal Care

Healthy adult male Wister rats each weighting 150-200g (four to six weeks old) were obtained from Breeding Laboratory, King Saud University, Riyadh, Saudi Arabia. The animals were labeled by identifying ear notches,

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housed in clean cages, and placed in the animal care room. Ethical guidelines for animal care were followed.

Effect of magnesium chloride and sodium fluoride on different Hyp fractions in rat kidneys

The following groups of rats were studied (each consisting of 4-6 rats) (i) normal rats (Control group, n = 4 - 6 rats); (ii) rats injected with $MgCl_2$ through intraperitoneal route 30 mg/kg body weight dose ($MgCl_2$ treated group); (iii) rats injected with NaF through intraperitoneal route 10 mg/kg body weight dose (NaF treated group); (iv) rats injected with $MgCl_2$ through intraperitoneal route 30 mg/kg body weight followed by NaF 10 mg/kg body weight through intraperitoneal route 30 minutes after $MgCl_2$ injection ($MgCl_2$ + NaF treated group).

Preparation of the sample

Rats were killed by carbon dioxide asphyxiation 24 hours after the final injection. The kidneys were dissected out, cleared of adhering tissues and weighed. The kidneys were then homogenized in normal saline (10% W/V) and the homogenate was used for Hyp determination as described below.

Extraction of Free, Peptide- and Protein-bound Hydroxyproline

Free and protein-bound Hyp was extracted by the method of Varghese *et al*. (1981) with slight modification. Briefly, 0.5ml of the homogenate was treated with 3 X 2 ml portion of re-rectified absolute alcohol and centrifuged at 3000rpm for 10min. The supernatants were pooled and evaporated to dryness. The residue was dissolved in suitable amount of distilled water and an aliquot of the extract was used for estimation of free Hyp. The peptide-bound Hyp was determined after alkaline hydrolysis of the ethanol extractable fraction. The pellets were dissolved in distilled water and an aliquot of the extract was used for determination of protein-bound Hyp. The free Hyp fraction was measured on an aliquot of the ethanol extracted residue without alkali hydrolysis, whereas the peptide-bound Hyp was measured after alkaline hydrolysis. The precipitate obtained on ethanol treatment of the homogenate was subjected to alkali hydrolysis to determine protein-bound Hyp. Further details about the extraction of Hyp fractions have been described previously (Siddiqi *et al*., 2000). Hyp was determined in different fractions as described in the later section.

Extraction of Soluble- and Insoluble-Collagen Hyp

Soluble- and insoluble-collagen Hyp were extracted by the method of Kivirikko *et al*. (1965). Briefly, the tissue samples were homogenized (4ml/g tissue) in a cold 0.45 M sodium chloride. The homogenate was extracted at 4^0C for 24 hours with occasional stirring, followed by centrifugation at 13000rpm for 1hour. The supernatants obtained were precipitated with 4 volumes of a cold ethanol and were centrifuged twice with 80% ethanol, twice with absolute alcohol, twice with ether and twice with warm ethanol-ether (1:2). The residues were gelatinized with distilled water at 120^0C for 3 hours and after filtration a sample of gelatine solution was used for soluble-collagen Hyp estimation as described below.

The precipitates obtained after the above centrifugation were washed 3 times with 0.45 M NaCl and twice with distilled water, after which they were extracted with absolute ethanol, ether and ethanol-ether and gelatinized as above. A sample of gelatine solution was used for insoluble-collagen Hyp estimation as described in the following section.

Determination of Hydroxyproline Concentration

Hyp was measured by the modified alkaline hydrolysis method of Reddy and Enwemeka (1996). Briefly, an aliquot of the sample was added into NaOH (2 N final concentration) and the aliquot was hydrolyzed by heating in a boiling water bath for about 3-4 hours. An aliquot of 56 mM chloramine-T reagent was added to the hydrolyzed sample and oxidation was allowed to proceed at room temperature for 25min. Then an aliquot of 1M Ehrlich's reagent (p-dimethylaminobenzaldehyde) was added to the oxidized sample and the chromophore was developed by incubating the samples at 65^0C for 20 minutes. The absorbance was read at 550 nm using an Ultrospec 2000 UV/visible spectrophotometer (Pharmcia Biotech Ltd, Science Park, Cambridge, England). The Hyp concentration in the samples was calculated from the standard curve of Hyp. Further details about the optimization, linearity, specificity, precision and reproducibility of the method have been described previously (Siddiqi *et al.,* 2000).

Determination of Total Collagen

Total collagen content was calculated from Hyp concentration assuming that Hyp constitutes 12.5% of total collagen (Edwards and O'Brien, 1980).

Statistical Analysis

Each sample was run in duplicate. The Hyp content was expressed as mean ± SD µg/gram wet tissue, for n = 4-6 rats. Hyp levels between groups were compared using one way ANOVA analysis followed by Tukey's for multiple comparison test. Values were considered significant if $p < 0.05$. Statistical analysis was performed by means of InStat® package for personal computers (GraphPad ™ Software, Inc., San Diego, USA).

RESULTS

Table 1 shows the effect of $MgCl_2$ and NaF on protein content of rat kidneys. $MgCl_2$ and NaF alone caused a significant decrease of 70% ($p<0.001$) and 24% ($p<0.01$) respectively in kidney protein content when compared to control rats. Combined treatment of $MgCl_2$ and NaF also caused a decrease of 63% ($p<0.001$) in the kidney protein content when compared to control group of rats.

Table 1. Effect of $MgCl_2$ and NaF treatment on protein content of rat kidneys.

Experimental groups	Kidney protein (mg/gram tissue)
Control	150.1 ± 37.5
$MgCl_2$	45.61 ± 2.04***
NaF	113.7 ± 22.64*
$MgCl_2$ + NaF	54.93 ± 2.88***

Rats were injected sodium fluoride (10 mg/kg body weight) and magnesium chloride (30 mg/kg body weight) through intraperitoneal route.
The rats were injected with sodium fluoride 30 minutes after magnesium chloride administration.
The animals were sacrificed 24 hours after the sodium fluoride treatment.
*P<0.05 as compared to control group (Tukey's multiple comparison test).
***P<0.001 as compared to control group (Tukey's multiple comparison test).

Table 2 shows the effect of $MgCl_2$ and NaF on various Hyp fractions in rat kidneys. $MgCl_2$ treatment of control rats caused a significant decrease of all the Hyp fractions of rat kidneys ($p < 0.001$) except protein-bound Hyp. NaF treatment also caused a significant decrease in free, peptide- bound and total Hyp concentration in rat kidneys ($p <0.001$). NaF treatment however caused no significant change ($P > 0.05$) in protein bound Hyp fractions in rat kidneys. Administration of $MgCl_2$ before NaF resulted in a tendency towards restoration of parameters altered by $MgCl_2$ or NaF towards normal level but did not completely restore them to normal levels.

Figure 1 shows the effect of $MgCl_2$ and NaF on total collagen in rat kidneys. $MgCl_2$ and NaF alone caused a significant decrease in total collagen ($p < 0.001$) in rat kidneys when compared to control rats. $MgCl_2$ injection before NaF injection caused an increase in total collagen when compared to $MgCl_2$ and NaF alone but it did not restore total collagen to normal level.

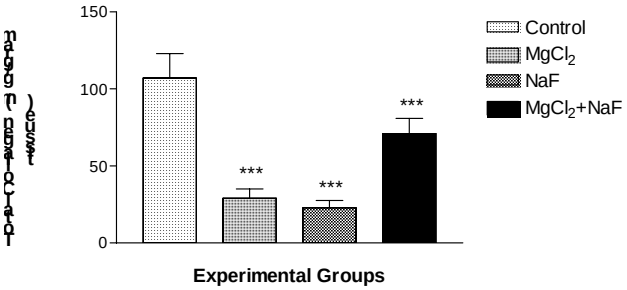

Fig. 1. Effect of magnesium chloride and sodium fluoride on total collagen content of rat kidneys.

Rats were injected magnesium chloride (30 mg/kg body weight) and sodium fluoride (10mg/kg body weight) through intraperitoneal route.
The rats were injected with sodium fluoride 30 minutes after magnesium chloride administration.
The animals were sacrificed 24 hours after the sodium fluoride treatment.
***P<0.01 as compared to 5 mg/kg body weight group (Tukey's multiple comparision test).

Figure 2 shows the effect of $MgCl_2$ and NaF on soluble collagen Hyp in rat kidneys. There was a significant decrease ($p < 0.001$) in soluble collagen Hyp in the

Table 2. Effect of $MgCl_2$ and NaF treatment on various hydroxyproline fractions in rat kidneys.

Experimental Groups	Free Hyp (µg/gm fresh tissue)	Hydroxyproline Fractions		Total Hyp (mg/gm fresh tissue)
		Peptide –bound Hyp (mg/gm fresh tissue)	Protein-bound Hyp (mg/gm fresh tissue)	
Control	329.3 ± 54.65	11.29 ± 0.88	1.04 ± 0.15	12.67 ± 0.96
$MgCl_2$	149.6 ± 22.77***	1.99 ± 0.47***	1.73 ± 0.17***	3.87 ± 0.48***
NaF	125.3± 15.94***	1.61 ± 0.91***	1.26 ± 0.23 ^{ns}	3.44 ± 0.79 ***
$MgCl_2$ + NaF	218.1 ± 11.16***	6.67 ± 1.23***	2.41 ± 0.33***	9.19 ± 1.21***

Rats were injected sodium fluoride (10 mg/kg body weight) and magnesium chloride (30 mg/kg body weight) through intraperitoneal route.
The rats were injected with sodium fluoride 30 minutes after magnesium chloride administration.
The animals were sacrificed 24 hours after the sodium fluoride treatment.
^{ns} Not siginificant as compared to control group (Tukey's multiple comparision test).
***P <0.001 as compared to control group (Tukey's multiple comparision test).

kidneys of all the experimental groups viz., $MgCl_2$ alone, NaF alone and $MgCl_2$ plus NaF treated groups when compared to control rats.

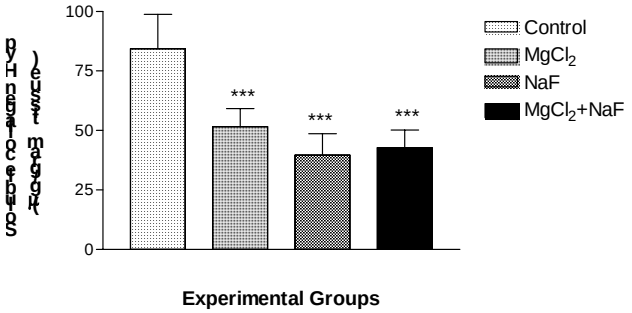

Fig. 2. Effect of magnesium chloride and sodium fluoride on soluble collagen hydroxyproline in rat kidneys.

Rats were injected sodium fluoride (10mg/kg body weight) and magnesium chloride (30mg/kg body weight) through intraperitoneal route.
The rats were injected with sodium fluoride 30 minutes after magnesium chloride administration.
The animals were sacrificed 24 hours after the sodium fluoride treatment.
***P<0.01 as compared to 5mg/kg body weight group (Tukey's multiple comparison test).

Figure 3 shows the effect of $MgCl_2$ and NaF on insoluble collagen Hyp in rat kidneys. Among all the groups studied only in the group of rats treated with NaF alone, there was a significant ($p < 0.01$) decrease in insoluble collagen Hyp in the kidneys when compared to control rats. $MgCl_2$ alone or with NaF caused no significant change ($p>0.05$) in insoluble collagen Hyp in rat kidneys.

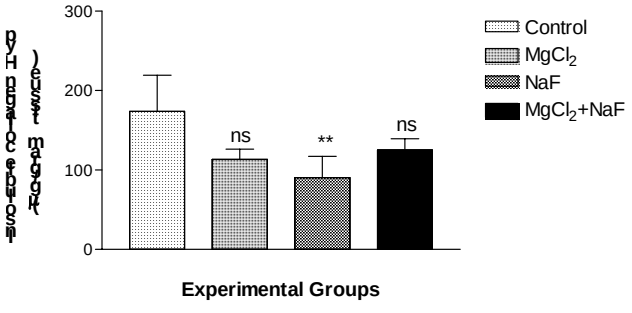

Fig. 3. Effect of magnesium chloride and sodium fluoride on insoluble collagen hydroxyproline in rat kidneys.

Rats were injected sodium fluoride and magnesium chloride through intraperitoneal route.
The rats were injected with sodium fluoride 30 minutes after magnesium chloride administration.
The animals were sacrificed 24 hours after the sodium fluoride treatment.
[ns]Not significant as compared to control group (Tukey's multiple comparison test);
**P<0.01 as compared to 5mg/kg body weight group (Tukey's multiple comparision test).

DISCUSSION

Fluoride is useful in preventing dental caries but excessive intake of fluoride can be toxic. The first noticeable signs of excessive exposure to fluoride are discoloration of the enamel. Abnormalities in mineralization processes affect by and large the osteoarticular system and are associated with changes in the density and structure of the bone presenting as irregular mineralization of the osteoid. Fluorine compounds also act on the organic part of supporting tissues, including collagen and other proteins, and on cells of the connective tissue. These interactions reduce the content of collagen proteins, modify the structure and regularity of collagen fibers and induce mineralization of collagen (Machoy-Makrzynska, 2004).

The kidneys are paired bean-shaped organs located on both sides of the spinal column. The kidneys perform a variety of functions for the body, the most important being removal of unwanted substances from the plasma, homeostasis of the body's water, electrolyte and acid/base status and participation in endocrine regulation. The amount of collagen in the kidney depends on factors like the species of the animal, its age and the presence of disease. In general collagen forms only a small proportion of the renal mass about 2% of the dry weight of renal cortex of adult rats (Weiss and Jayson, 1982) and this may be due to the presence of an active collagenolytic mechanism in the kidney (Weiss and Jayson, 1982). Nevertheless, collagen is of great physiological importance as a support for the renal parenchyma and as a component of the basement membrane. Magnesium chloride administered thirty minutes before sodium fluoride has been shown to increase the LD_{50} for fluoride from 76 to 104 mg/kg body weight (Luoma *et al.*, 1984). In the present study both $MgCl_2$ as well as NaF were found to decrease the protein content in the kidneys. Earlier studies of Birkner *et al.* (2000) have found disturbance in protein metabolism after acute dose of sodium fluoride. Though the most common medium used to study fluoride toxicity is urine, fluoride has been reported to cause renal tissue damage and disrupt renal function (Zabulyte *et al.*, 2007). Oxidative stress has been reported to be one of the factors in the pathogenesis of sodium fluoride (Ranjan *et al.*, 2009). Doses of NaF and $MgCl_2$ caused a decrease in peptide- bound Hyp. Hyp is excreted by the kidneys as small peptides (Adams and Frank, 1980). In the present study NaF treatment caused a decrease in total collagen. The degraded collagen may be excreted in the form of peptide-bound Hyp causing a decrease in peptide-bound Hyp fraction. These results are in agreement with earlier studies (Sharma, 1982[a]) which have demonstrated that fluoride interferes with the collagen biosynthesis resulting in decreased collagen content (in terms of hydroxyproline). Therefore the decrease in collagen content of kidneys of NaF treated

animals may be either due to decreased synthesis or increased degradation by collagenase (Machoy – Mokrzynska, 2004).

In the present study soluble collagen Hyp was found to be more susceptible to the effects of both $MgCl_2$ as well as NaF. Both $MgCl_2$ and NaF caused a decrease in soluble collagen Hyp in rat kidneys. Earlier studies of Prockop (1964) have shown that there exists at least 3 pools of body collagen with half lives of about 1day, 5days and 50-100 days. The first two of these represent the soluble collagen fractions, i.e. fractions containing collagen not yet aggregated to insoluble-collagen fiber and the third pool, the insoluble collagen. In the present study the soluble collagen Hyp appeared to be susceptible to degradation by $MgCl_2$ and NaF. This may be due to the fact that it has still not aggregated to form insoluble collagen fibers. Studies of Sharma (1982[b]) have shown that fluoride interferes with collagen crosslinking. Studies by the same author have also demonstrated that NaF interferes with maturation and normal metabolism of tissue collagen. The insoluble collagen Hyp was affected only by NaF treatment alone. Administration of $MgCl_2$ before NaF restored the altered insoluble collagen Hyp to near normal levels. This appears to be the only protective effect of $MgCl_2$ on NaF induced change in kidney collagen.

CONCLUSION

Therefore the present study concludes that though $MgCl_2$ has been reported to be protective against the toxic effect of NaF it has no significant effect on NaF induced changes in kidney Hyp/collagen. However administration of $MgCl_2$ before NaF restored insoluble collagen hydroxyproline which was altered by NaF to near normal levels.

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