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Original Article

Urea adulteration alters raw milk composition and induces renal tissue damage: a molecular and histopathological study

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Abstract

This study investigates the impact of urea adulteration in raw milk on milk quality, safety, and renal tissue integrity, addressing a significant public health concern, particularly in low-to-middle-income countries where over 76.6% of milk samples have compromised quality and 77.89% are adulterated. Raw milk samples were analyzed for quality parameters, adulterants, and the presence of synthetic by-products using FTIR. In vivo studies in mice were conducted to assess the histopathological effects of urea-adulterated milk on renal and liver tissues. Results revealed that urea adulteration leads to significant alterations in milk composition, including changes in fat content from 5.73% to 0.6% in boiled milk, solids-not-fat from 9.11% to 12.84%, protein from 3.61% to 5.28%, and lactose content from 3.81% to 5.57%, alongside the formation of synthetic compounds such as lactose monohydrate and polyvinyl stearate. Histological examination of mice kidneys exposed to urea-adulterated milk demonstrated morphological, inflammatory, degenerative, congestive, and necrotic changes. This study highlights the cellular-level damage induced by urea adulteration, emphasizing its potential to induce neoplastic changes in renal tissues and underscoring the need for stringent monitoring of milk quality to safeguard public health.

Keywords: Milk, Adulteration, Urea, Milk quality, Synthetic products, Milk safety.

1. Introduction

Raw milk is considered the ideal food for both infants and adults due to the availability of a variety of nutrients. It is also considered a complete diet as it consists of almost all components like protein, carbohydrates, fats, vitamins, and minerals [1]. Milk is a pale liquid produced by mammal's mammary glands and is the primary source of nutrition for infants. Early lactation milk contains colostrum, which helps to build the immune system [2]. Milk is the normal, clean, and pure secretion obtained from the udders of a healthy cow, buffalo, goat, or sheep. It may either be available as raw (fresh) milk or processed milk [3].

The milk secretion in the udder of a cow is secreted in the alveoli of the mammary gland. The compositional constituents of milk, like lactose, fat, protein, minerals, and vitamins, can pass through the cell membrane from the blood stream through diffusion, active transport, and passive transport mechanisms [4]. Environmental factors as well as the animal's genetic makeup, nutritional status, and lactation phases influence milk composition and quality [5]. Milk is 87.00% water, and the main milk sugar lactose makes up 4 –5%, followed by 3% proteins, 3-4% lipids, 0.80% minerals and lastly 0.10% vitamins [6].

Global milk production has increased to 906 million tons [7], while Asians contribute to milk production with 378 million tons [8] and Pakistan milk production with

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57,722 thousand tons [9]. Pakistan has a total of Milk transported via intermediaries, commonly known locally as 'dhodhies' [10].67.00 million cattle and buffaloes, 89.00 million sheep and goats, and 0.20 million camels [11]. In developing countries such as Pakistan, Brazil, India, and China, milk adulteration has been commonly observed [12]. Such milk is watered to increase volume. To maintain its composition, starch, flour, urea, cane sugar, vegetable oil, etc. are added as chemical adulterants [13].

Some of the main causes of milk adulteration include a demand and supply gap, milk being readily perishable, and being more expensive as compared to other commodities. Furthermore, lack of adulteration regulation and detection makes it easier to sell adulterated milk, particularly in low and middle-income countries [14]. Recent studies in Pakistan show that about 80.0% of sold milk is adulterated [15]. Unscrupulous milk producers boost their profit margins by diluting milk, extracting valuable components such as cream and fat, and adding cheap additives to balance the quality characteristics of milk [16].

Some of the adulterants added in milk alter the composition of milk in a dishonest way, such as urea, which is added to enhance the desirable qualities of milk such as solid not fat (SNF), milk nitrogen and melamine content [17]. Unfortunately, some of the milk adulterants have serious long-term health consequences [18]. Melamine consumption at levels higher than the recommended limit can cause renal failure and death in newborns [19]. Milk with adulterants may cause health issues like gastrointestinal problems such as gastritis and bowel inflammation, diarrhea, and diabetes [20]. Furthermore, carbonate and bicarbonates may impair hormone signaling, which controls development and reproduction [21].

Given the widespread practice of urea adulteration in raw milk and its potential health risks, this study aimed to comprehensively assess the impact of urea on milk quality and safety at the cellular level. Specifically, we sought to: (1) evaluate the changes in milk composition and the formation of novel synthetic by-products resulting from urea adulteration, (2) investigate the histopathological effects of consuming urea-adulterated milk on murine renal and liver tissues, and (3) elucidate the molecular mechanisms underlying any observed tissue damage. By combining compositional analysis with in vivo toxicological assessments, this research provides critical insights into the health implications of milk adulteration, highlighting the urgent need for effective monitoring and regulatory measures.

2. Materials and Methods

2.1. Study design and sample collection

A prospective study was conducted to assess the impact of urea adulteration on raw milk composition and quality. A total of 30 raw milk samples, along with control samples, were collected using standardized methods from various sources including milk collectors, distributors/retailers, middlemen (locally known as 'dhodhies'), and end consumers at the Nutrition Division of NIH.

2.2. Stability study of raw milk

A preliminary stability study was performed to determine the shelf life of raw milk stored at 2–8°C. Fresh raw milk samples remained stable for 4 days, showing negligible decreases in fat (from 5.0% to 4.98%), solid-not-fat (SNF) (from 8.03% to 7.97%), and total solids (from 13.03% to 12.95%).

2.3. Physicochemical analysis of milk samples

Physical and chemical parameters including acidity/pH, total solids, total fat, SNF, lactose, moisture, total protein, and ash content [22] were analyzed to evaluate the initial quality of raw milk samples.

2.4. Detection of added urea in milk

Added urea was detected using a colorimetric reaction with p-Dimethyl Amino Benzaldehyde (DMAB) reagent. In a low acidic medium, this reaction produces a distinct yellow color indicating the presence of urea adulteration above 70 mg/100 ml, with a minimum detection limit of 0.2%.

2.5. Preparation of in-house urea-standard milk samples

To study the effects of urea on milk quality and safety, milk samples were artificially adulterated with urea (Sigma Aldrich 5378) following the Material Safety Data Sheet (MSDS) and Limit of Detection (LOD) guidelines. Urea was added at concentrations of 0.424 mg/250 ml, 0.212 mg/250 ml, and 0.106 mg/250 ml representing double, normal, and half dilutions, respectively.

2.6. Quality analysis of urea-treated milk samples

The urea-adulterated milk samples were analyzed for changes in total solids, total fat, SNF, lactose, and total protein. Additionally, boiled milk samples (100°C, 24 hours storage at 2–8°C) were dried and analyzed using Fourier Transform Infrared Spectroscopy (FTIR) to detect molecular and compositional alterations.

2.7. Fourier transform infrared spectroscopy (FTIR) analysis

FTIR spectra were normalized and baseline-corrected before analysis. Samples were placed in microtitre plates and scanned in the mid-infrared region to assess chemical bond vibrations, providing detailed information on the structural and compositional changes induced by urea adulteration.

2.8. *In Vivo* study on milk safety using albino mice

An in vivo study was conducted using albino mice (15–35 g) to evaluate the toxicological effects of urea-adulterated milk. Mice were acclimatized for one week and divided into control and test groups. Urea doses in milk (0.424 mg/dose, 0.212 mg/dose, and 0.106 mg/dose) were administered according to body weight (average 25 g) via enteral or parenteral routes.

2.9. Animal husbandry and experimental procedures

Mice were housed in standard cages, fed a diet containing fish meat, mustard, rice, sesame, and wheat, with filtered tube-well water provided ad libitum. After two inoculations per dose, mice were observed for 72 hours for any adverse effects.

2.10. Histopathological examination

After the observation period, mice were humanely sacrificed using chloroform exposure. Liver and renal tissues were collected, fixed, dehydrated, cleared, paraffin-

embedded, and sectioned. Tissue slides were stained with Hematoxylin and Eosin (H&E) for histopathological analysis to assess tissue damage caused by urea adulteration.

3. Results

Physical and chemical analysis of milk samples found 47.3% to be unsatisfactory. Out of the 110 samples, 94.2% reported decreased milk fat, 90.4% had reduced milk solids, and 75% had lowered specific gravity. Water was also added to milk along with other adulterants and analysis also showed 58 (52.7%) samples were found satisfactory with 41/58 (70.7%) samples having normal quality parameters showing natural raw milk or precisely prepared adulterated milk while 17/58 (29.3%) samples had exceptionally raised quality parameters as fat 16/17 (94.1%) samples, total solids 14/17 (82.4%) and specific gravity 12/17 (70.6%) of samples showed the addition of water as well as other adulterants. The assessment of the quality of fresh milk through physical and chemical parameters not only described the poor quality of fresh milk but also gave some clues regarding the addition of different sorts of adulterants (>76.6%).

The analysis of different adulterants in milk samples showed water in 148(77.89%) raw milk samples, Detergent in 62(32.9%), Cane Sugar 41(21.8%), Caustic Soda 32(16.8%), Sodium Salts 31(16.4%), Starch 21(11.1%), Formalin 18(9.4%), Urea 15(8.05%), Foreign Fat 12(6.4%), Hydrogen Peroxide 04(2.3%), Glucose 02(1.3%), Boric Acid 02(1.1%) and sulfate salts 02(1.1%) in raw milk samples. Quality assessments of fresh milk revealed widespread adulteration, with 77.89% of raw milk samples containing various adulterants and 2.63% showing signs of semi-synthetic composition.

The findings related to effects of added urea as adulterant on the composition of stored milk showed that the values of milk fat, SNF (Solid Not Fat), TS (Total Solids), protein, and lactose for the control are 5.73%, 9%, 14.74%, 3.76%, and 3.61%, respectively. For milk stored at temperatures between 2-8˚C, the corresponding values were 5.73%, 9.11%, 14.84%, 3.81%, and 3.61%. Boiled milk, on the other hand, exhibits values of 0.6%, 12.84%, 13.44%, 5.57%, and 5.28%, as depicted in Figure 1.

The results from the stored milk composition indicated the synthesis of certain synthetic products, which significantly influenced the quality parameters of milk samples. This led to an increase in concentrations of solid-not-fat, protein, and deceptively decrease in fat contents due to the presence of urea as adulterant. The synthetic products identified were:

1. Lactose Monohydrate
2. Polyvinyl Stearate
3. Urea

Histological examinations were conducted on two major organs, the liver and kidney, of experimental animals (mice), and are depicted in Figure 2. The effect of urea on liver tissues is illustrated through H & E staining. Figure 2 (A) serves as a representation of normal histology from the control group. Figure 2 (B) shows mild liver congestion, marked with a plus sign. Figure 2 (C) displays mild morphological changes and hyperchromasia, both marked with the indication of mild intensity, suggesting subtle alterations in cell structure and nuclear staining intensity. In summary, Figures provide insights into the histological effects of urea, on the liver. The observed changes include mild congestion, morphological changes, and hyperchromasia in response to urea.

A lethal index was developed to assess the lethality of each adulterant on mice liver and its potential impact on human health. . (+) represented the presence of the corresponding histological feature. (++) Indicated a higher degree or intensity of the feature. Mild suggested a mild or moderate presence of the feature. (-) Implied the absence of the specific histological feature.

Histological findings related to the safety status of raw milk treated with added urea as adulterant with double dilution showed only mild changes as shown in Table 1, with respect to morphological changes, Hyperchromasia

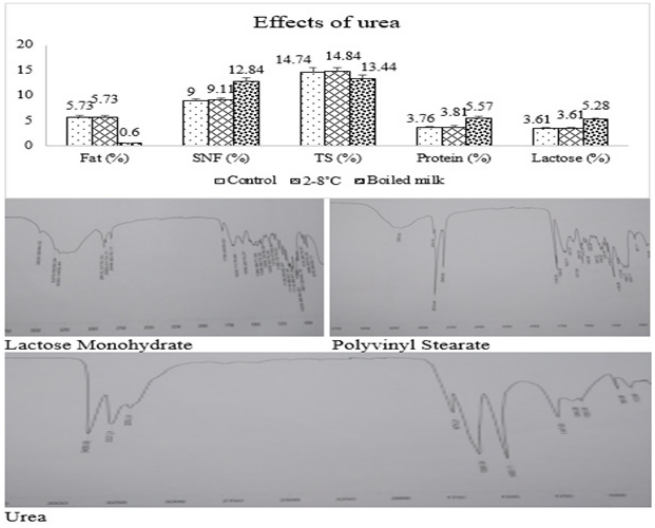

Fig. 1. Effects of added urea as adulterant on the composition of stored milk.

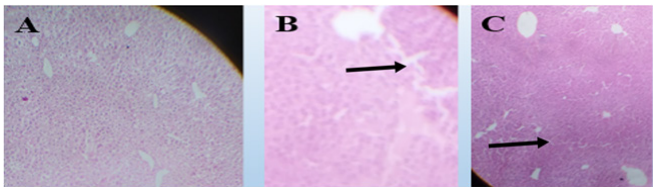

Fig. 2. Histological examinations were conducted on two major organs, the liver and kidney, of experimental animals (mice). The effect of urea on liver tissues is illustrated through H & E staining. Figure (A) serves as a representation of normal histology from the control group. Figure (B) shows mild liver congestion, marked with a plus sign. Figure (C) displays mild morphological changes and hyperchromasia, both marked with the indication of mild intensity, suggesting subtle alterations in cell structure and nuclear staining intensity.

Table 1. Physical and chemical characteristics of raw milk samples

Adulterants	Morphological changes	Hyperchromasia	Acute inflammatory changes	Chronic inflammatory changes	Congestion	Necrotic changes	Degenerative changes	Neoplastic changes
Urea	Mild	Mild	-	-	Mild	-	-	-

and Congestion in the liver induced by urea as adulterant while Inflammatory changes, Degenerative changes, Necrotic or Neoplastic changes do not show below by urea as adulterant on milk safety to albino mice and even on human health.

The effect of urea on mice kidney tissues is presented through H & E staining. Figure 3(A) provides a representation of normal histology from the control group. Figure 3(B) indicates the presence of kidney congestion, marked with a plus sign. Figure 3(C) shows morphological changes and hyperchromasia, both marked with plus signs, suggesting alterations in cell structure and increased staining intensity in nuclei. Figure 3(D) reveals acute inflammatory changes, marked with a plus sign, indicating an inflammatory response in the kidney tissues. Urea induces kidney congestion, morphological changes, hyperchromasia, and acute inflammatory changes.

A lethal index was developed to assess the lethality of each adulterant in mice kidneys and its potential impact on human health. (+) represented the presence of the corresponding histological feature. (++) Indicated a higher degree or intensity of the feature. Mild suggested a mild or moderate presence of the feature. (-) Implied the absence of the specific histological feature.

Histological findings related to the safety status of raw milk treated with added urea as adulterant with double dilution showed urea emerged as the most lethal adulterant, causing morphological changes, hyperchromasia, acute inflammatory responses, and moderate congestion, as shown in Table 2. The persistent presence of causative agents resulted in the development of chronic inflammatory areas characterized by fibrous tissue, presenting the potential for either benign or malignant outcomes.

4. Discussion

Economically motivated adulteration involves the addition of vegetable protein, milk from different species, whey, and watering [18]. Various studies have identified common milk adulterants, including water or water with contaminants, sodium carbonate, sodium bicarbonate, caustic soda, formalin, urea, detergents, ammonium sulfate, boric acid, benzoic acid, salicylic acid, hydrogen peroxide, starch, sugars, and melamine [23]. Our study aimed to assess the impact of urea adulteration on various aspects of milk quality.

Added urea-treated milk samples showed that there was an increase in the concentration of protein, SNF (solid not fat), and lactose. The natural urea content of milk is 18-40mg/dl, which indicates adequate protein content in the cow's diet. Urea is mainly added to milk to increase its nitrogen content and thereby increasing its protein content. The total protein content of milk is estimated by multiplying with conversion factor of 6.25. Additionally, urea addition also increases milk whiteness and its shelf life which is favorable for milk vendors and increases milk sales. The permissible urea limit that is deemed safe for consumption is 70mg/dl, and amounts more than this can result in necrosis and degeneration of the liver and kidneys [24]. Urea increases SNF in milk, which decreases fat and cream sensory properties while enhancing the odor of skimmed milk. SNFs include non-fat contents in milk such as protein, phosphorus, calcium, riboflavin, and other water-soluble vitamins. Urea increases SNF in milk as a way to mimic protein presence. When SNFs are increased, they falsely increase the level of protein content in milk. The level of SNF in milk is tightly regulated and a minimum of 3.25% milk fats and 8.25% SNF is allowed. SNF levels above this mark are considered hazardous to health and result in renal and gastrointestinal tract dysfunction. Therefore, SNF is used as an indicator of milk adulteration and quality by various authorities [25]. Milk urea nitrogen levels fluctuate during lactation, peaking during 90-120 days and then also at the end. Conversely, urea nitrogen content in adulterated milk is abnormally high and does not follow the natural milk urea fluctuation pattern. Furthermore, urea nitrogen decreases milk lactose levels and adulterated milk with unnaturally high urea levels may disrupt lactose balance. This can further impact sweetness and fermentation properties of the milk [26].

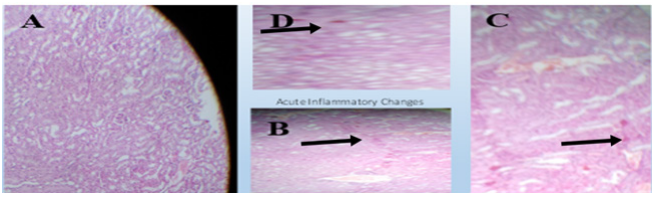

Fig. 2. Effect of urea on mice kidney (H & E staining) A: Normal histology from control, B: Congestion (+), C: Morphological changes (+) & Hyperchromasia (+), D: Acute inflammatory Changes (+).

Added urea-treated milk samples also showed a decrease in the concentration of total solids and fat. This is similar to an Egyptian study, which reported the mean milk solids concentration in their urea adulterated sample to be 10.04± 0.13 for raw milk and 10.07± 0.073 for UHT milk. Both values were lower than the minimum Egyptian standards of milk solids; not less than 11.25%. Additionally, the study also found mean fat concentration in raw milk to be 3.01± 0.098, 3.10± 0.058 for UHT milk, and 56.99±2.757 for thick cream samples. The low-fat content was attributed to lower forage consumption, leading to decreased acetate and butyrate, both important fat precursors in milk. Furthermore, fat is often skimmed off from the milk further reducing its concentration in the milk samples. Urea is then added to give milk a rich and thick appearance when in fact it is low in fat and SNFs as well. Removing fat from milk also decreases its fat-soluble vitamin content, thereby further reducing its overall quality [27].

The observed changes in hepatic tissues included mild congestion, morphological changes, and hyperchromasia in response to urea. Increased blood urea level leads to compromised liver function, and can progress to protein energy malnutrition. Elevated urea levels are correlated

Table 2. Prevalence of various adulterants detected in raw milk samples

Adulterants	Morphological changes	Hyperchromasia	Acute inflammatory changes	Chronic inflammatory changes	Congestion	Necrotic changes	Degenerative changes	Neoplastic changes
Urea	+	+	+	-	+	-	-	-

with hepatic fibrosis and can lead to liver carcinogenesis [28]. Further studies found urea in milk to result in degeneration and necrosis of hepatocytes as well as lymphoid follicle formation. Additionally, renal damage was also observed, with perirenal tissues undergoing fatty changes and necrosis. Glomerulitis and leukocytic infiltration were also observed in 48 rats who were fed urea-adultered milk for 28 days. In some cases, the hepatic and renal damage was severe enough to cause infant deaths, in those consuming urea derivatives in skim and non-fat milk. Ingestion of more than 70mg/dl of urea adultered milk can cause renal failure, liver damage, carcinogenesis, and obstruction of the urinary tract as well as bleeding in the gastrointestinal tract [29].

Furthermore, the synthesis of new products was identified through FTIR, including lactose monohydrate, polyvinyl stearate, and urea. Lactose monohydrate is a crystallized form of milk sugar commonly used as a filler in medications and added to various packaged foods, baked goods, and infant formulas for its sweetening and stabilizing properties. Widely considered safe, lactose monohydrate typically does not cause symptoms in individuals who are lactose intolerant [30]. The formation of lactose monohydrate occurs when crystallized lactose is hydrated with one molecule of water. This form of lactose is the most common commercially available solid lactose and can be used to enhance lactose concentration, as observed in some adulterant-treated stored milk compositions [31].

FTIR findings indicated that lactose monohydrate exhibits stretching vibrations of the hydroxyl group in the range of 3600-3200/cm, with a weak band at 1650/cm corresponding to the hydroxyl group of water. Additionally, the band in the range of 1200-1070/cm signifies the asymmetric stretching vibration of C-O-C in glucose and galactose. Significant peaks are observed at 3520/cm and 920/cm, consistent with lactose monohydrate. The FTIR spectrum, resulting from the vibration of various structural groups, generates multiple bands related to protein, lipids, carbohydrates, and nucleic acids. Specifically, a band around 3295/cm represents N-H stretching, associated with proteins. A band around 3000-2800/cm represents C-H stretching, primarily from CH3 & CH2 groups, indicative of lipids. A band around 1700-1500/cm represents Amide I & Amide II. A band around 1500-1000/cm represents functional groups of carbohydrates and nucleic acids [32].

There are two major limitations of our study. Firstly, we were only able to collect n = 30 milk samples. This study would have had greater impact had we collected 100 or more milk samples. Secondly, the collection of milk samples was only limited to rural Islamabad. It would have been very interesting to determine the quality of milk samples collected from other adjacent areas and then compare the results.

Considering these limitations of our study, we recommend that future researchers replicate this study on a bigger sample size and collect milk samples from all the possible adjoining areas and then conduct a comparative analysis.

In the present study, the added urea as an adulterant not only caused the quality compromised substandard milk but also caused changes in the composition of milk due to formulation of lactose monohydrate and polyvinyl stearate by the reaction of added urea with main constituents of milk. In the same way, on milk safety using a lethal index, it was found that urea emerged as the least lethal adulterant, causing mild changes in mice liver, while urea was identified as the most lethal for mice kidneys, inducing morphological changes, hyperchromasia, acute inflammatory changes, and moderate congestion. These findings in mice organs may parallel potential effects on human health, with observed conditions such as necrosis, degeneration, and inflammation. The study suggests that the persistence of causative agents may lead to chronic inflammatory areas such as fibrous tissue, which could be either benign or show neoplastic changes.

Conflict of interest
The authors report no conflict of interest

Consent for publications
All the authors have read and approved the final manuscript for publication.

Ethics approval and consent to participate
The ethical approval of the study was obtained from the Institutional Ethical Committee, vide letter no. PMAS-AAUR/IEC/119.

Availability of data and material
The authors declare that they embedded all data in the submitted manuscript.

Author contributions
Tanveer Ibrahim: Conceptualization, Literature Search, Data Collection, Writing Original Draft; Feroza Hamid Wattoo: Conceptualization, Supervision, Review & Editing; Muhammad Hamid Sarwar Wattoo: Conceptualization, Supervision, Final Proofreading; Asif Ahmad: Conceptualization, Data Analysis; Muhammad Sheeraz Ahmad: Conceptualization, Data Analysis; Hussain Ali: Conceptualization, Data Collection
Syed Hassan Bin Usman Shah: Data Analysis; Rida Fatima Saeed: Writing original draft; Umar Farooq: Conceptualization, Data curation; Juweria Abid: Review & Editing; Sajeela Akram: Data curation; Zoha Imtiaz Malik: Literature Search, Review & Editing; Abdul Momin Rizwan Ahmad: Literature Search, Review & Editing

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