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Nitrate in drinking water and risk of colorectal cancer in Yogyakarta, Indonesia

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ABSTRACT

Nitrate concentration in well water in Yogyakarta, Indonesia, and its surroundings tended to increase rapidly from time to time, and it may be associated with an elevated risk for several types of cancer. The purpose of this study was to examine the association between nitrate in drinking water and colorectal cancer (CRC) risk occurrence. A case-control study was conducted in Yogyakarta Special Province. Pathologically confirmed 75 CRC patients and 75 controls were consulted and their individual well water was sampled and examined for nitrate concentrations. Logistic regression analysis was conducted to establish the association between nitrate and CRC risk development. There was a significant correlation between nitrate in drinking water and CRC occurrence, and this value was relatively stable after being adjusted for protein intake, smoking history, age, and family history of cancer. These findings demonstrated that the risk of CRC development was fourfold among those with >10 years of nitrate exposure from well water compared with those with ≤10 years of nitrate exposure. Consequently, a significant association between nitrate in drinking water and occurrence of CRC in Yogyakarta was established.

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Introduction

Yogyakarta is one of the provinces in Indonesia with the highest prevalence of cancer. Cancer prevalence in Yogyakarta is approximately 4.1%. This prevalence rate exceeds the national prevalence rate (1.4%) (Balitbangkes, 2013). In addition, from one of the major hospitals in the province, Dr. Sardjito Hospital, data from 2011 to 2014 showed colorectal cancer (CRC) rose from 3.7 per 100,000 in 2011 to 4.5 per 100,000 population in 2014.

Nitrate is one of the chemical constituents that has been increasing in concentration in well water in Yogyakarta, Indonesia, over the last three decades. When comparing nitrate in well water from 1991 to 2014, the nitrate levels rose rapidly. In 1991, Sudarmadji (1991) reported the mean nitrate concentration in Yogyakarta Municipality was 3.47 mg/L. Five years later, Smith et al. (1999) investigated nitrate levels in well water in the city of Yogyakarta, precisely in Kotagede District during 1994–1996, where the threshold allowed by the WHO (50 mg/L) had been exceeded. Several studies conducted in Yogyakarta and its surrounding from 2005 to 2014 indicated the concentration of nitrate in well water was approximately threefold higher than permitted by the WHO levels (Basuki, 2011; Oudone, 2014; Putra, 2010; Wiguna, 2014). Putra (2010) reported an elevation of nitrate in well water in the city of Yogyakarta and the surrounding areas attributed to inadequate sanitation. In fact of significant concern, 70% individuals residing in Yogyakarta were using well water as a water source (Balitbangkes, 2008).

Nitrates may be present in humans originating through two pathways, endogenous and exogenous. Endogenous nitrate results from the process of nitrite circulation and other nitrogen oxides (NO) derived from metabolism in the blood and tissues (Kawanishi et al., 2006). Exogenous nitrates enter through exposure from the environment via ingestion from food, imbibition of water, inhalation, or a combination (Mensinga et al., 2003).

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Nitrate is a nontoxic substance (Mensinga et al., 2003). Toxicity occurs when nitrates are reduced to nitrites by commensal bacteria in the oral cavity (Gilchrist et al., 2010; Lundberg et al., 2008; Mensinga et al., 2003). Furthermore, on a regular basis in the stomach, nitrite spontaneously decomposes into NO, peroxynitrite ($ONOO^-$), and dinitrogen trioxide (N_2O_3) (Lundberg et al., 2008). These compounds are known as reactive nitrogen species (RNS). The existence of RNS in the human body serves two functions. NO produced endogenously functions to kill pathogenic bacteria, stimulate mucosal blood flow, and produce mucus, thereby enhancing the protection of the stomach (Lundberg et al., 2008). On the other hand, the existence of excessive RNS with unbalanced antioxidant production may produce adverse effects (Goodman et al., 2008). NO reacts with superoxide ($O_2^{\bullet-}$) to form $ONOO^-$, resulting in a highly reactive 8-nitroguanine and nitrative DNA damage (Hiraku et al., 2010; Thanan et al., 2015), leading to the development of chronic inflammation. Inflammation may also be initiated by physical exposure to chemicals and immunological factors (Hiraku et al., 2010; Kawanishi et al., 2006). In this case, the inflammation may occur because of exposure to nitrates in drinking water. To date, there has been no apparent method to distinguish the source of nitrate in the body.

Nitrate is a precursor in the formation of nitrosamines. The International Agency for Research on Cancer (IARC) classified nitrate as a probable human carcinogen (class 2A) that may form N-nitroso compounds (NOCs) through endogenous nitrosation (IARC, 2010). Since nitrate is synthesized in the gastrointestinal tract (GIT), bacterial metabolism in the GIT and cancer development may be affected by diet. Bacterial metabolism of dietary protein containing amino acids releases NOC (Hughes and Rowland, 2000). Inflammatory conditions such as inflammatory bowel disease (IBD) might also enhance the formation of NOC (Thanan et al., 2015). In addition, smoking also produces NOC (Lu et al., 2016).

Studies related to the relationship between nitrate in drinking water and CRC risk demonstrated inconclusive results (Chang et al., 2010; Chen et al., 2005; Kuo et al., 2007; Yang et al., 2007; De Roos et al., 2003; Espejo-Herrera et al., 2016; McElroy et al., 2008). The aim of this study was to correlate pathologically confirmed case control of CRC patients with nitrate content originating from each patient's water-well, taking into account different confounding factors that might influence the results.

Material and Methods

Study Design and Population

The study was carried out in the city of Yogyakarta, and two neighboring districts, Sleman and Bantul. An unmatched case-control design was used. Cancer cases and controls were recruited from patients at Dr. Sardjito General Hospital Yogyakarta recorded from January 1, 2014 to February 29, 2016. Cancer cases were patients who were categorized as C18-C21 based on International Classification of Disease 10th revision codes (ICD-10 codes), and confirmed pathologically as adenocarcinoma, and with no IBD history. Controls were colon biopsy patients who had been confirmed pathologically as non-neoplastic (ICD-10 codes: K51, and K62). Inclusion criteria for participants were residents of the city of Yogyakarta, Sleman, or Bantul District for a minimum of three years, minimum age of 18, and willing to be engaged in this study by signing the consent form. Informed consent was obtained from all individual participants in the study. Exclusion criteria for participants were patients who had another cancer type or metastasis, home address has changed, did not consume well water, or passed away at the date of study. Subject recruitment began by sorting out data from medical records. Participants were selected by residential address. Patients who had an incomplete address or had other cancers or died when hospitalized were excluded from the potential participant list. The process of selecting subjects is illustrated in Figure 1.

Sample and Data Collection

Data and samples from well water were collected by trained interviewers. Participants were interviewed between October 2015 and March 2016.

Nitrate in Well Water

The water samples were collected in the rainy season (February to March 2016) according to guidelines of

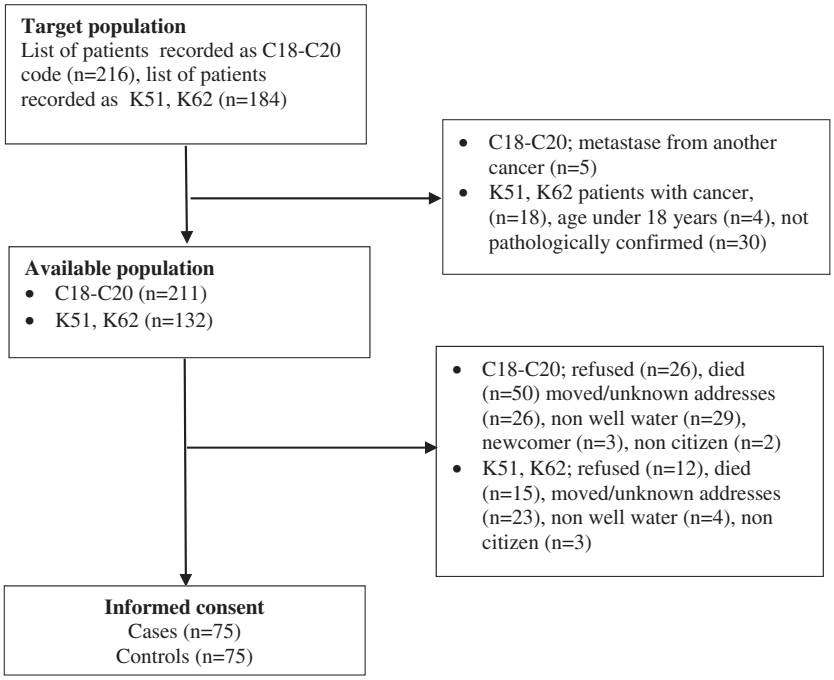

Figure 1. Schematic of participant recruitment.

SNI 6989.58:2008 (Indonesian National Standard on well water sampling). The water samples were analyzed in Water Chemical Laboratory of Major Center of Environmental Health Engineering and Disease Control (MCEHE-DC) Yogyakarta using APHA 2012 standard, section 4500-NO_3 to establish the nitrate levels. Nitrate was classified based on the WHO threshold for nitrate in drinking water (≤50 mg/L and >50 mg/L as NO_3^-).

Estimation of Dietary Protein Intake

The 18 food items listed in the food frequency questionnaire (FFQ), which were classified as protein, were used to gather information regarding protein intake. Participants were asked to recall their eating habits one year prior to diagnosis. To avoid bias, their relatives who knew them well were asked to confirm the participants' eating habits. Participants were asked to recall the frequency of protein intake and their usual portion based on household size. The household size data were converted to gram using a grocery list exchanger (Waspadji et al., 2011) before they were processed using modified *Nutrisurvey 2007* software (EBISpro, Germany). The results of *Nutrisurvey 2007* analysis were compared with recommended dietary allowances per person based on gender and age, which is issued by the Ministry of Health of the Republic of Indonesia (Kementerian Kesehatan, 2013) in order to obtain the information regarding individual protein intake. Because only two participants had "sufficient" category for protein intake, "sufficient" and "less" categories were merged to become the "less-sufficient" category. Therefore, the protein variable was classified into two categories (less-sufficient and more).

Socio-Demographic and Clinical Related Data

Personal history questionnaires (PHQs) were used to collect socio-demographic data, such as age when diagnosed, date of birth, gender, education, and family income. Information regarding diabetes, family history of cancer, smoking history, and a lifetime residential history was collected using PHQ. The primary source of drinking water was documented to ensure subjects used well water as a major source for their daily needs.

All data were classified into two categories. Age was classified into below 50 and 50 years and above (Brenner et al., 2007), genders were classified into female and male, and education was classified into more than 12 years and 12 years or less based on the program from the Government of Indonesia. Family

income was classified based on the average of county/city minimum wages (below and above average). Length of nitrate exposure was classified >10 years and ≤10 years (De Roos et al., 2003). Diabetes and family history of cancer were classified by "No" and "Yes", whereas smoking history was classified by "Never" and "Ever".

Statistical Analysis

Unadjusted odds ratios (ORs) and 95% confidence intervals (CIs) were used to determine the strength of the relationship in the bivariate analysis. The bivariate analysis using Chi-square was conducted on the independent variable and all variables that allegedly affected the CRC risk. The result of this analysis was used to perform multivariate analysis using logistic regression in order to obtain the most ideal model on the incidence of CRC. The stepwise method in logistic regression was employed to determine the most suitable model to analyze the relationship between nitrate and CRC. The predicted variables associated with cancer were included in the model. Known variables such as the risk of CRC were used in the model regardless of the level of statistical significance. Stratification analysis of the association of nitrate concentration in well water and CRC was made based on the length of exposure. Stata version 12 was utilized to perform statistical analysis. Two-sided p value ≤0.05 was considered as statistically significant.

Results

Characteristics of Study Participants

Table 1 presents the socio-demographic and clinical characteristics of study participants. Gender distribution was similar between cases and controls. Sleman was the main contributor of participants recruited (48%), whereas Yogyakarta was the least contributor (21%), but with similar distribution between cancer cases and controls. Controls were significantly more educated than cancer cases. No marked difference between cancer cases and controls was found for family income and length of exposure with nitrate. Cancer cases with confirmed diagnosis as C18 represented the majority (49%), compared with C19 (13%) and C20 (37%).

Table 1. Socio-demographic and clinical characteristics of the study participants.

	Cases (n=75)	Controls (n=75)
Variables	n (%)	n (%)
Gender		
Male	38 (50.67)	38 (50.67)
Female	37 (49.33)	37 (49.33)
Place of living		
Yogyakarta	16 (21.33)	8 (10.67)
Sleman	36 (48.00)	46 (61.33)
Bantul	23 (30.67)	21 (28.00)
Level of education*		
≤12 years	40 (53.33)	23 (30.67)
>12 years	35 (46.67)	52 (69.33)
Family income (Rp)		
Mean ± SD	3089200 ± 2423113	3253107 ± 2333887
≤Rp1,362,700	14 (18.67)	18 (24.00)
>Rp1,362,700	61 (81.33)	57 (76.00)
Length of exposure (years)		
Mean ± SD	29.53 ± 15.84	28.71 ± 18.75
>10 years	64 (85.33)	59 (78.67)
≤10 years	11 (14.67)	16 (21.33)
Clinical status (ICD-10 code)		
C18	37 (49.33)	
C19	10 (13.33)	
C20	28 (37.33)	
K51		60 (80.00)
K62		15 (20.00)

*= Significant from control $p \leq 0.05$.

Nitrate in Well Water and Risk of CRC

Table 2 shows the correlation of nitrate exposure from well water, smoking habit, age, and cancer family history, with risk of CRC occurrence. Difference in mean nitrate concentrations in well water between cancer cases and controls was significant. Controls consumed significantly more protein than cancer cases. Compared with controls, cancer cases tended to smoke more. Cancer cases were significantly older than controls, and more likely to have a family history of cancer and diabetes. There was an overall significant association of CRC occurrence with high nitrate in well water after nitrates were adjusted for confounding by protein intake, smoking history, age, family history of cancer, and diabetes. There was an inverse relationship between protein intake and CRC. There was no marked association between smoking history and CRC development, and between age and CRC. A significant association between family history of cancer and CRC development was found. A correlation between CRC with diabetes was not detected.

Table 2. Correlation of nitrate exposure from well water, smoking habit, age, family history of cancer, and risk of CRC.

Variables	Cases/Controls	Unadjusted[a] OR (95% CI)	Adjusted[b] OR (95% CI)	Cases/Controls	Adjusted[c] OR (95% CI)	Cases/Controls	Adjusted[c] OR (95% CI)
			Overall	Length of Exposure >10 years		Length of Exposure ≤ 10 years	
Nitrate							
Mean ± SD	30.51 ± 27.91/20.26 ± 15.05						
>50 mg/L	19/8	2.842 (1.080–8.047)	2.820 (1.075–7.395)*	16/4	4.312 (1.319–14.098)*	3/4	1.405 (0.144–13.677)
≤50 mg/L	56/67			48/55		8/12	
Protein intake							
Mean ± SD	53.32 ± 27.56/71.50 ± 40.25						
More	55/36	0.336 (0.159–0.701)	0.316 (0.148–0.677)*				
Less-sufficient	20/39						
Smoking History							
Ever	29/23	1.425 (0.687–2.967)	1.367 (0.649–2.875)	27/18	1.575 (0.721–3.439)	2/5	0.273 (0.023–3.185)
Never	46/52			37/41		11/9	
Age							
Mean ± SD	56.12 ± 11.47/50.16 ± 13.72						
≥50 years	55/40	2.406 (1.152–5.067)	1.887 (0.901–3.950)	49/38	1.429 (0.625–3.271)	6/2	12.476 (1.507–103.27)*
<50 years	20/35			15/21		5/14	
Family history of cancer							
Yes	16/8	2.271 (0.839–6.561)	3.233 (1.150–9.087)*	53/5	2.670 (0.852–8.369)	3/3	4.783 (0.439–52.149)
No	59/67			51/54		13/8	
Diabetes							
Yes	4/1	4.169 (0.397–208.002)	5.559 (0.559–55.297)				
No	71/4						

a = Odds Ratio (OR) in bivariate analysis using Chi-square analysis.
b = Odds Ratio (OR) was calculated using logistic regression, adjusted for protein intake, smoking history, age, family history of cancer, and diabetes.
c = Odds Ratio (OR) was calculated using logistic regression, adjusted for smoking history, family history of cancer, and age.
* = two-sided p value of ≤0.05 was considered statistically significant.

Based on stratification analysis, the risk of CRC occurrence attributed to nitrate became higher for subjects with exposure to nitrate longer than 10 years compared with less exposure. After adjusting for smoking history, age, and family history of cancer, the correlation between CRC development and exposure to well water nitrate was further increased. Protein and diabetes variables were not included in the model for stratification analysis. The protein variable in our research was assumed not as a nitrate source due to the observations that average intake of nitrate was lower than the recommended dietary allowances, whereas the diabetes variable was not possible to be determined due to insufficient number of subjects with diabetes.

Discussion

This study examined the risk relationship between CRC occurrence and exposure to nitrate in drinking water. In our study, water-well was sampled only once. However, exposure to nitrates for each individual was presumed to be on a chronic daily basis. Nitrate has a conservative ion, which does not readily react physically, chemically, and biologically during its travels through the groundwater flow (Freeze and Cherry, 1979). Soil structure and its porosity, and water supply from precipitation and irrigation influence nitrate leaching (Ritter et al., 2002). It is worthwhile noting that Yogyakarta has a relatively high rainfall, approximately 300 to >500 mm/month (BMKG, 2016) and an annual recharge of groundwater approximately ≥600 mm/acre (Putra, 2007). The condition of rainfall and recharge of groundwater in Yogyakarta make it difficult to decrease the nitrate levels in groundwater, especially if the nitrate sources are not eliminated. Since the 1980s, nitrate concentration in groundwater in Yogyakarta and its surroundings tended to increase rapidly from time to time due to improper sanitation (Putra, 2011). In our study it was found that there were 25 of 27 wells with high nitrate (>50 mg/L) located less than 10 m from the septic tank (data not shown).

Data demonstrated an association between nitrate exposure in drinking water containing nitrate >50 mg/L with elevated risk of CRC development. This result is consistent with studies conducted by Espejo-Herrera et al. (2016) in Spain and Italy. A correlation between nitrate in drinking water and CRC occurrence was noted, especially for rectal cancer in China (Chen et al., 2005) and Taiwan (Kuo et al., 2007; Chang et al., 2010). McElroy et al. (2008) found the association was limited to proximal colon cancer for rural women in Wisconsin, USA, and the association was stronger among those who were exposed to nitrate from drinking water for more than 10 years. However, De Roos et al. (2003), who studied a population in Iowa, did not find a relationship between consumption water with high nitrate for more than 10 years and risk of CRC development. However, it should be noted that De Roos et al. (2003) observed that the risk pertained to the exposure of nitrate (>10 years with average nitrate >5 mg/L as nitrate-N) and CRC was elevated among the subgroup with low vitamin C intake and high meat intake. When compared to the study of De Roos et al. (2003), our study showed 25% of cases were with high nitrate exposure, while only 7% of cancer cases were detected in the Iowa investigation. Thus, differences in these findings may be attributed to a larger population being exposed to high nitrate levels in drinking water in our study.

Data suggest that the source of nitrosamines through cigarette smoking allegedly associated with CRC occurrence did not show sufficient evidence (Table 2). The protein consumed by participants in this study served as a protective factor. Generally meat may serve as a nitrate source from food and meat consumption; however, in Indonesia meat consumption is lower compared with other countries in Southeast Asia (Saleh, 2016). In addition, this study did not distinguish protein intake by source. Therefore, nitrate and nitrogen from diet could not be taken into account. Thus, subjects in this study may be presumed as pathologically confirmed with nitrate exposure from drinking water.

Data demonstrated that consumption of high nitrate in drinking water was not the only risk for CRC development. It was found that a family history of cancer played some significant role and presented as high a risk for CRC development. Although diabetes appeared not to be related with CRC development, subjects with diabetes tend to develop CRC to a greater extent than patients who have no history of diabetes (Deng

et al., 2012). Individuals with smoking history and aged population also appeared to display a higher risk for CRC occurrence.

The Joint FAO/WHO Expert Committee on Food Additives set the Acceptable Daily Intake (ADI) for nitrate from 0 to 3.7 mg/kg body weight (FAO/WHO, 2002). Data on the amount of nitrates consumed by the participants through drinking water were not available, but the amount of nitrate from drinking water intake can be estimated. Assuming an average weight of 60 kg and the record of participants consuming water containing nitrate of 50 mg/L with as much as 2 L per day, then the intake of nitrates from drinking water is approximately 1.67 mg/kg body weight per day. This value is still within the range of ADI and does not include nitrate intake from food. Thus, the standard of nitrate in drinking water needs to be examined further to determine whether a maximum concentration of 50 mg/L (as nitrate) is required to be lowered. The importance of the standard of nitrate in drinking water is essential to be reassessed because of the impact of nitrate not only on CRC, but for risk of cancers occurring, including non-Hodgkin's lymphoma in Nebraska (Ward et al., 1996), thyroid cancer and hypothyroidsm in older women in Iowa (Ward et al., 2010), childhood brain tumors in Taiwan (Weng et al., 2011), gastric cancer in Valencia, Spain (Morales-Suarez-Varela et al., 1995), methemoglobinemia among infants and children in Morocco (Sadeq et al., 2008), and insulin-dependent diabetes mellitus among children in Colorado (Kostraba et al., 1992).

A limitation in this study was the use of only 75 cancer cases and 75 controls; however, the results showed an association between nitrate concentration in drinking water and CRC occurrence, raising concerns regarding the high nitrate concentrations in drinking water. Further studies with larger populations are required to confirm these findings. Evidence indicated that the CRC risk occurred when consuming high nitrate in well water, especially for long periods of time. Several steps may be taken to prevent the emergence of this CRC risk. First, monitoring well water quality should be conducted regularly by the government through primary health centers. Second, the prevention of nitrate pollution in well water may be accomplished through proper sanitation. Third is avoid consuming well water if the water is contaminated by high nitrate.

Ethical Approval

This research was approved by the Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine Universitas Gadjah Mada-Dr. Sardjito General Hospital (Ref: KE/FK/955/EC/2015).

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Nitrate in drinking water and risk of colorectal cancer in Yogyakarta Indonesia

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Nitrate in drinking water and risk of colorectal cancer in Yogyakarta, Indonesia

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ABSTRACT

Nitrate concentration in well water in Yogyakarta, Indonesia, and its surroundings tended to increase rapidly from time to time, and it may be associated with an elevated risk for several types of cancer. The purpose of this study was to examine the association between nitrate in drinking water and colorectal cancer (CRC) risk occurrence. A case-control study was conducted in Yogyakarta Special Province. Pathologically confirmed 75 CRC patients and 75 controls were consulted and their individual well water was sampled and examined for nitrate concentrations. Logistic regression analysis was conducted to establish the association between nitrate and CRC risk development. There was a significant correlation between nitrate in drinking water and CRC occurrence, and this value was relatively stable after being adjusted for protein intake, smoking history, age, and family history of cancer. These findings demonstrated that the risk of CRC development was fourfold among those with >10 years of nitrate exposure from well water compared with those with ≤10 years of nitrate exposure. Consequently, a significant association between nitrate in drinking water and occurrence of CRC in Yogyakarta was established.

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Introduction

Yogyakarta is one of the provinces in Indonesia with the highest prevalence of cancer. Cancer prevalence in Yogyakarta is approximately 4.1%. This prevalence rate exceeds the national prevalence rate (1.4%) (Balitbangkes, 2013). In addition, from one of the major hospitals in the province, Dr. Sardjito Hospital, data from 2011 to 2014 showed colorectal cancer (CRC) rose from 3.7 per 100,000 in 2011 to 4.5 per 100,000 population in 2014.

Nitrate is one of the chemical constituents that has been increasing in concentration in well water in Yogyakarta, Indonesia, over the last three decades. When comparing nitrate in well water from 1991 to 2014, the nitrate levels rose rapidly. In 1991, Sudarmadji (1991) reported the mean nitrate concentration in Yogyakarta Municipality was 3.47 mg/L. Five years later, Smith et al. (1999) investigated nitrate levels in well water in the city of Yogyakarta, precisely in Kotagede District during 1994–1996, where the threshold allowed by the WHO (50 mg/L) had been exceeded. Several studies conducted in Yogyakarta and its surrounding from 2005 to 2014 indicated the concentration of nitrate in well water was approximately threefold higher than permitted by the WHO levels (Basuki, 2011; Oudone, 2014; Putra, 2010; Wiguna, 2014). Putra (2010) reported an elevation of nitrate in well water in the city of Yogyakarta and the surrounding areas attributed to inadequate sanitation. In fact of significant concern, 70% individuals residing in Yogyakarta were using well water as a water source (Balitbangkes, 2008).

Nitrates may be present in humans originating through two pathways, endogenous and exogenous. Endogenous nitrate results from the process of nitrite circulation and other nitrogen oxides (NO) derived from metabolism in the blood and tissues (Kawanishi et al., 2006). Exogenous nitrates enter through exposure from the environment via ingestion from food, imbibition of water, inhalation, or a combination (Mensinga et al., 2003).

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Nitrate is a nontoxic substance (Mensinga et al., 2003). Toxicity occurs when nitrates are reduced to nitrites by commensal bacteria in the oral cavity (Gilchrist et al., 2010; Lundberg et al., 2008; Mensinga et al., 2003). Furthermore, on a regular basis in the stomach, nitrite spontaneously decomposes into NO, peroxynitrite ($ONOO^-$), and dinitrogen trioxide (N_2O_3) (Lundberg et al., 2008). These compounds are known as reactive nitrogen species (RNS). The existence of RNS in the human body serves two functions. NO produced endogenously functions to kill pathogenic bacteria, stimulate mucosal blood flow, and produce mucus, thereby enhancing the protection of the stomach (Lundberg et al., 2008). On the other hand, the existence of excessive RNS with unbalanced antioxidant production may produce adverse effects (Goodman et al., 2008). NO reacts with superoxide ($O_2^{\bullet -}$) to form $ONOO^-$, resulting in highly reactive 8-nitroguanine and nitrative DNA damage (Hiraku et al., 2010; Thanan et al., 2015), leading to the development of chronic inflammation. Inflammation may also be initiated by physical exposure to chemicals and immunological factors (Hiraku et al., 2010; Kawanishi et al., 2006). In this case, the inflammation may occur because of exposure to nitrates in drinking water. To date, there has been no apparent method to distinguish the source of nitrate in the body.

Nitrate is a precursor in the formation of nitrosamines. The International Agency for Research on Cancer (IARC) classified nitrate as a probable human carcinogen (class 2A) that may form N-nitroso compounds (NOCs) through endogenous nitrosation (IARC, 2010). Since nitrate is synthesized in the gastrointestinal tract (GIT), bacterial metabolism in the GIT and cancer development may be affected by diet. Bacterial metabolism of dietary protein containing amino acids releases NOC (Hughes and Rowland, 2000). Inflammatory conditions such as inflammatory bowel disease (IBD) might also enhance the formation of NOC (Thanan et al., 2015). In addition, smoking also produces NOC (Lu et al., 2016).

Studies related to the relationship between nitrate in drinking water and CRC risk demonstrated inconclusive results (Chang et al., 2010; Chen et al., 2005; Kuo et al., 2007; Yang et al., 2007; De Roos et al., 2003; Espejo-Herrera et al., 2016; McElroy et al., 2008). The aim of this study was to correlate pathologically confirmed case control of CRC patients with nitrate content originating from each patient's water-well, taking into account different confounding factors that might influence the results.

Material and Methods

Study Design and Population

The study was carried out in the city of Yogyakarta, and two neighboring districts, Sleman and Bantul. An unmatched case-control design was used. Cancer cases and controls were recruited from patients at Dr. Sardjito General Hospital Yogyakarta recorded from January 1, 2014 to February 29, 2016. Cancer cases were patients who were categorized as C18-C21 based on International Classification of Disease 10th revision codes (ICD-10 codes), and confirmed pathologically as adenocarcinoma, and with no IBD history. Controls were colon biopsy patients who had been confirmed pathologically as non-neoplastic (ICD-10 codes: K51, and K62). Inclusion criteria for participants were residents of the city of Yogyakarta, Sleman, or Bantul District for a minimum of three years, minimum age of 18, and willing to be engaged in this study by signing the consent form. Informed consent was obtained from all individual participants in the study. Exclusion criteria for participants were patients who had another cancer type or metastasis, home address has changed, did not consume well water, or passed away at the date of study. Subject recruitment began by sorting out data from medical records. Participants were selected by residential address. Patients who had an incomplete address or had other cancers or died when hospitalized were excluded from the potential participant list. The process of selecting subjects is illustrated in Figure 1.

Sample and Data Collection

Data and samples from well water were collected by trained interviewers. Participants were interviewed between October 2015 and March 2016.

Nitrate in Well Water

The water samples were collected in the rainy season (February to March 2016) according to guidelines of

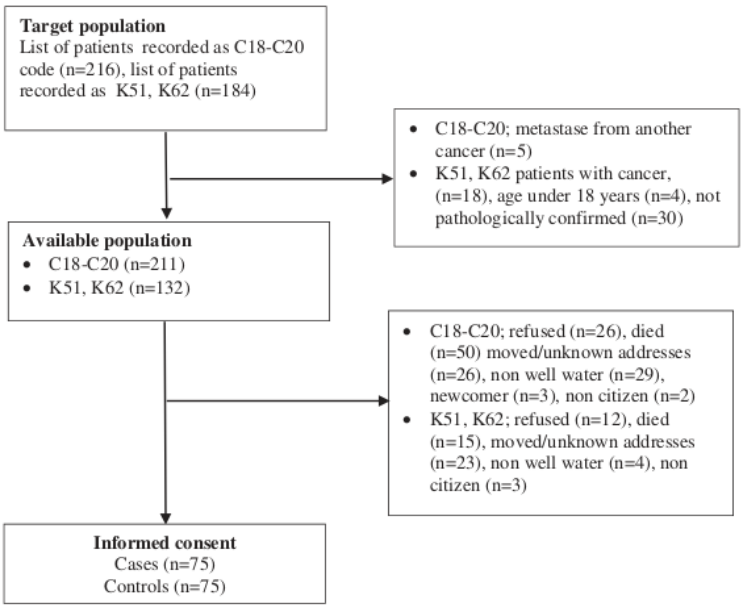

Figure 1. Schematic of participant recruitment.

SNI 6989.58:2008 (Indonesian National Standard on well water sampling). The water samples were analyzed in Water Chemical Laboratory of Major Center of Environmental Health Engineering and Disease Control (MCEHE-DC) Yogyakarta using APHA 2012 standard, section 4500-NO_3 to establish the nitrate levels. Nitrate was classified based on the WHO threshold for nitrate in drinking water (≤50 mg/L and >50 mg/L as NO_3^-).

Estimation of Dietary Protein Intake

The 18 food items listed in the food frequency questionnaire (FFQ), which were classified as protein, were used to gather information regarding protein intake. Participants were asked to recall their eating habits one year prior to diagnosis. To avoid bias, their relatives who knew them well were asked to confirm the participants' eating habits. Participants were asked to recall the frequency of protein intake and their usual portion based on household size. The household size data were converted to gram using a grocery list exchanger (Waspadji et al., 2011) before they were processed using modified *Nutrisurvey 2007* software (EBISpro, Germany). The results of *Nutrisurvey 2007* analysis were compared with recommended dietary allowances per person based on gender and age, which is issued by the Ministry of Health of the Republic of Indonesia (Kementerian Kesehatan, 2013) in order to obtain the information regarding individual protein intake. Because only two participants had "sufficient" category for protein intake, "sufficient" and "less" categories were merged to become the "less-sufficient" category. Therefore, the protein variable was classified into two categories (less-sufficient and more).

Socio-Demographic and Clinical Related Data

Personal history questionnaires (PHQs) were used to collect socio-demographic data, such as age when diagnosed, date of birth, gender, education, and family income. Information regarding diabetes, family history of cancer, smoking history, and a lifetime residential history was collected using PHQ. The primary source of drinking water was documented to ensure subjects used well water as a major source for their daily needs.

All data were classified into two categories. Age was classified into below 50 and 50 years and above (Brenner et al., 2007), genders were classified into female and male, and education was classified into more than 12 years and 12 years or less based on the program from the Government of Indonesia. Family

income was classified based on the average of county/city minimum wages (below and above average). Length of nitrate exposure was classified >10 years and ≤10 years (De Roos et al., 2003). Diabetes and family history of cancer were classified by "No" and "Yes", whereas smoking history was classified by "Never" and "Ever".

Statistical Analysis

Unadjusted odds ratios (ORs) and 95% confidence intervals (CIs) were used to determine the strength of the relationship in the bivariate analysis. The bivariate analysis using Chi-square was conducted on the independent variable and all variables that allegedly affected the CRC risk. The result of this analysis was used to perform multivariate analysis using logistic regression in order to obtain the most ideal model on the incidence of CRC. The stepwise method in logistic regression was employed to determine the most suitable model to analyze the relationship between nitrate and CRC. The predicted variables associated with cancer were included in the model. Known variables such as the risk of CRC were used in the model regardless of the level of statistical significance. Stratification analysis of the association of nitrate concentration in well water and CRC was made based on the length of exposure. Stata version 12 was utilized to perform statistical analysis. Two-sided p value ≤0.05 was considered as statistically significant.

Results

Characteristics of Study Participants

Table 1 presents the socio-demographic and clinical characteristics of study participants. Gender distribution was similar between cases and controls. Sleman was the main contributor of participants recruited (48%), whereas Yogyakarta was the least contributor (21%), but with similar distribution between cancer cases and controls. Controls were significantly more educated than cancer cases. No marked difference between cancer cases and controls was found for family income and length of exposure with nitrate. Cancer cases with confirmed diagnosis as C18 represented the majority (49%), compared with C19 (13%) and C20 (37%).

Table 1. Socio-demographic and clinical characteristics of the study participants.

Variables	Cases (n=75) n (%)	Controls (n=75) n (%)
Gender		
Male	38 (50.67)	38 (50.67)
Female	37 (49.33)	37 (49.33)
Place of living		
Yogyakarta	16 (21.33)	8 (10.67)
Sleman	36 (48.00)	46 (61.33)
Bantul	23 (30.67)	21 (28.00)
Level of education*		
≤12 years	40 (53.33)	23 (30.67)
>12 years	35 (46.67)	52 (69.33)
Family income (Rp)		
Mean ± SD	3089200 ± 2423113	3253107 ± 2333887
≤Rp1,362,700	14 (18.67)	18 (24.00)
>Rp1,362,700	61 (81.33)	57 (76.00)
Length of exposure (years)		
Mean ± SD	29.53 ± 15.84	28.71 ± 18.75
>10 years	64 (85.33)	59 (78.67)
≤10 years	11 (14.67)	16 (21.33)
Clinical status (ICD-10 code)		
C18	37 (49.33)	
C19	10 (13.33)	
C20	28 (37.33)	
K51		60 (80.00)
K62		15 (20.00)

*= Significant from control $p \leq 0.05$.

Nitrate in Well Water and Risk of CRC

Table 2 shows the correlation of nitrate exposure from well water, smoking habit, age, and cancer family history, with risk of CRC occurrence. Difference in mean nitrate concentrations in well water between cancer cases and controls was significant. Controls consumed significantly more protein than cancer cases. Compared with controls, cancer cases tended to smoke more. Cancer cases were significantly older than controls, and more likely to have a family history of cancer and diabetes. There was an overall significant association of CRC occurrence with high nitrate in well water after nitrates were adjusted for confounding by protein intake, smoking history, age, family history of cancer, and diabetes. There was an inverse relationship between protein intake and CRC. There was no marked association between smoking history and CRC development, and between age and CRC. A significant association between family history of cancer and CRC development was found. A correlation between CRC with diabetes was not detected.

Table 2. Correlation of nitrate exposure from well water, smoking habit, age, family history of cancer, and risk of CRC.

Variables	Cases/Controls	Unadjusted[a] OR (95% CI)	Adjusted[b] OR (95% CI) Overall	Cases/Controls Length of Exposure >10 years	Adjusted[c] OR (95% CI) Length of Exposure >10 years	Cases/Controls Length of Exposure ≤ 10 years	Adjusted[c] OR (95% CI) Length of Exposure ≤ 10 years
Nitrate							
Mean ± SD	30.51 ± 27.91/20.26 ± 15.05						
>50 mg/L	19/8	2.842 (1.080–8.047)	2.820 (1.075–7.395)*	16/4	4.312 (1.319–14.098)*	3/4	1.405 (0.144–13.677)
≤50 mg/L	56/67			48/55		8/12	
Protein intake							
Mean ± SD	53.32 ± 27.56/71.50 ± 40.25						
More	55/36	0.336 (0.159–0.701)	0.316 (0.148–0.677)*				
Less-sufficient	20/39						
Smoking History							
Ever	29/23	1.425 (0.687–2.967)	1.367 (0.649–2.875)	27/18	1.575 (0.721–3.439)	2/5	0.273 (0.023–3.185)
Never	46/52			37/41		11/9	
Age							
Mean ± SD	56.12 ± 11.47/50.16 ± 13.72						
≥50 years	55/40	2.406 (1.152–5.067)	1.887 (0.901–3.950)	49/38	1.429 (0.625–3.271)	6/2	12.476 (1.507–103.27)*
<50 years	20/35			15/21		5/14	
Family history of cancer							
Yes	16/8	2.271 (0.839–6.561)	3.233 (1.150–9.087)*	53/5	2.670 (0.852–8.369)	3/3	4.783 (0.439–52.149)
No	59/67			51/54		13/8	
Diabetes							
Yes	4/1	4.169 (0.397–208.002)	5.559 (0.559–55.297)				
No	71/4						

a = Odds Ratio (OR) in bivariate analysis using Chi-square analysis.
b = Odds Ratio (OR) was calculated using logistic regression, adjusted for protein intake, smoking history, age, family history of cancer, and diabetes.
c = Odds Ratio (OR) was calculated using logistic regression, adjusted for smoking history, family history of cancer, and age.
* = two-sided *p* value of ≤0.05 was considered statistically significant.

Based on stratification analysis, the risk of CRC occurrence attributed to nitrate became higher for subjects with exposure to nitrate longer than 10 years compared with less exposure. After adjusting for smoking history, age, and family history of cancer, the correlation between CRC development and exposure to well water nitrate was further increased. Protein and diabetes variables were not included in the model for stratification analysis. The protein variable in our research was assumed not as a nitrate source due to the observations that average intake of nitrate was lower than the recommended dietary allowances, whereas the diabetes variable was not possible to be determined due to insufficient number of subjects with diabetes.

Discussion

This study examined the risk relationship between CRC occurrence and exposure to nitrate in drinking water. In our study, water-well was sampled only once. However, exposure to nitrates for each individual was presumed to be on a chronic daily basis. Nitrate has a conservative ion, which does not readily react physically, chemically, and biologically during its travels through the groundwater flow (Freeze and Cherry, 1979). Soil structure and its porosity, and water supply from precipitation and irrigation influence nitrate leaching (Ritter et al., 2002). It is worthwhile noting that Yogyakarta has a relatively high rainfall, approximately 300 to >500 mm/month (BMKG, 2016) and an annual recharge of groundwater approximately ≥600 mm/acre (Putra, 2007). The condition of rainfall and recharge of groundwater in Yogyakarta make it difficult to decrease the nitrate levels in groundwater, especially if the nitrate sources are not eliminated. Since the 1980s, nitrate concentration in groundwater in Yogyakarta and its surroundings tended to increase rapidly from time to time due to improper sanitation (Putra, 2011). In our study it was found that there were 25 of 27 wells with high nitrate (>50 mg/L) located less than 10 m from the septic tank (data not shown).

Data demonstrated an association between nitrate exposure in drinking water containing nitrate >50 mg/L with elevated risk of CRC development. This result is consistent with studies conducted by Espejo-Herrera et al. (2016) in Spain and Italy. A correlation between nitrate in drinking water and CRC occurrence was noted, especially for rectal cancer in China (Chen et al., 2005) and Taiwan (Kuo et al., 2007; Chang et al., 2010). McElroy et al. (2008) found the association was limited to proximal colon cancer for rural women in Wisconsin, USA, and the association was stronger among those who were exposed to nitrate from drinking water for more than 10 years. However, De Roos et al. (2003), who studied a population in Iowa, did not find a relationship between consumption water with high nitrate for more than 10 years and risk of CRC development. However, it should be noted that De Roos et al. (2003) observed that the risk pertained to the exposure of nitrate (>10 years with average nitrate >5 mg/L as nitrate-N) and CRC was elevated among the subgroup with low vitamin C intake and high meat intake. When compared to the study of De Roos et al. (2003), our study showed 25% of cases were with high nitrate exposure, while only 7% of cancer cases were detected in the Iowa investigation. Thus, differences in these findings may be attributed to a larger population being exposed to high nitrate levels in drinking water in our study.

Data suggest that the source of nitrosamines through cigarette smoking allegedly associated with CRC occurrence did not show sufficient evidence (Table 2). The protein consumed by participants in this study served as a protective factor. Generally meat may serve as a nitrate source from food and meat consumption; however, in Indonesia meat consumption is lower compared with other countries in Southeast Asia (Saleh, 2016). In addition, this study did not distinguish protein intake by source. Therefore, nitrate and nitrogen from diet could not be taken into account. Thus, subjects in this study may be presumed as pathologically confirmed with nitrate exposure from drinking water.

Data demonstrated that consumption of high nitrate in drinking water was not the only risk for CRC development. It was found that a family history of cancer played some significant role and presented as high a risk for CRC development. Although diabetes appeared not to be related with CRC development, subjects with diabetes tend to develop CRC to a greater extent than patients who have no history of diabetes (Deng

et al., 2012). Individuals with smoking history and aged population also appeared to display a higher risk for CRC occurrence.

The Joint FAO/WHO Expert Committee on Food Additives set the Acceptable Daily Intake (ADI) for nitrate from 0 to 3.7 mg/kg body weight (FAO/WHO, 2002). Data on the amount of nitrates consumed by the participants through drinking water were not available, but the amount of nitrate from drinking water intake can be estimated. Assuming an average weight of 60 kg and the record of participants consuming water containing nitrate of 50 mg/L with as much as 2 L per day, then the intake of nitrates from drinking water is approximately 1.67 mg/kg body weight per day. This value is still within the range of ADI and does not include nitrate intake from food. Thus, the standard of nitrate in drinking water needs to be examined further to determine whether a maximum concentration of 50 mg/L (as nitrate) is required to be lowered. The importance of the standard of nitrate in drinking water is essential to be reassessed because of the impact of nitrate not only on CRC, but for risk of cancers occurring, including non-Hodgkin's lymphoma in Nebraska (Ward et al., 1996), thyroid cancer and hypothyroidsm in older women in Iowa (Ward et al., 2010), childhood brain tumors in Taiwan (Weng et al., 2011), gastric cancer in Valencia, Spain (Morales-Suarez-Varela et al., 1995), methemoglobinemia among infants and children in Morocco (Sadeq et al., 2008), and insulin-dependent diabetes mellitus among children in Colorado (Kostraba et al., 1992).

A limitation in this study was the use of only 75 cancer cases and 75 controls; however, the results showed an association between nitrate concentration in drinking water and CRC occurrence, raising concerns regarding the high nitrate concentrations in drinking water. Further studies with larger populations are required to confirm these findings. Evidence indicated that the CRC risk occurred when consuming high nitrate in well water, especially for long periods of time. Several steps may be taken to prevent the emergence of this CRC risk. First, monitoring well water quality should be conducted regularly by the government through primary health centers. Second, the prevention of nitrate pollution in well water may be accomplished through proper sanitation. Third is avoid consuming well water if the water is contaminated by high nitrate.

Ethical Approval

This research was approved by the Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine Universitas Gadjah Mada-Dr. Sardjito General Hospital (Ref: KE/FK/955/EC/2015).

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