

The effect of nitrite food preservatives added to se'i meat on the expression of wild-type p53 protein

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Research Article

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Abstract: This research was conducted using beef extracted from Kupang (se'i meat), Indonesia. Se'i meat is a locally found food where the preferred mode of preparation is smoking the beef with the preservation using nitrites. Nitrite can cause health-related problems such as cancer. This research was carried out using a true experimental method with a complete randomized design with the aim of analyzing the effect of meat administration on the expression of wild-type p53 protein in colon cells of Balb/c mice as an indicator of carcinogenesis. The measurement of p53 is to observe the increase in the over-capacity of p53 expression in the colon cell as a result of decrease in wild-type protein p53. This research provides scientific information about the effect of giving se'i meat on the expression of wild-type p53 in cells of Balb/c mice as an indicator of carcinogenesis. A total of 36 male mice of Balb/c strain weighing 23.8 g were divided into four groups classified as samples (P1, P2 and P3) and control (K), which were taken from modern and home industries in the city of Kupang. The results showed that consumption of nitrite-preserved beef se'i (traditional smoked meat) increased the p53 protein expression in colon cells of Balb/c strain male mice, and the least significant difference test also showed that there were differences in wild-type p53 protein expression among the four groups: P1 (mice that have been given the standard food, drinking water and se'i meat that contains no nitrite) has an average of 142 expressions, which is higher than that of P3 (mice that have been given the standard food, drinking water and se'i meat containing nitrite which come from the home industry) which has an

average of 106.55 expressions and is higher than that of K (mice that have been given the standard food and drinking water) which has the total average of expression of about 78.11 expressions. The benefit of this research is to gain the scientific information about the effect of giving smoked meat on the expression of wild-type p53 in colon cells of Balb/c mice as a carcinogenic indicator.

Keywords: se'i, nitrite, wild-type p53 expression

1 Introduction

Promoting healthy living has been a challenge for a global society, and the need to increase nutritional consumption plays an important role in supporting lifestyles [1–3]. Meat is a nutritional food that is almost inseparable from human life; however, the quality and availability in the community are often not guaranteed. Meat contains proteins that are one of the important food substances for the body, such that they have functions such as cell growth, replacement of damaged cells and as fuel in the human body. Protein deficiency can cause health problems in humans [4]. One of the processed meat products commonly known throughout the world is smoked meat [5–8]. Almost all countries in the world have special meat preparations [9], including Indonesia, especially in the East Nusa Tenggara Province who also process beef in the city of Kupang. Due to its nutritional value, se'i meat (local name) is one of the processed smoked beef that has a high protein content of 30–32%, a fat content ranging from 0.8 to 0.92% and a high water content of 63%, causing it to be easily contaminated by microbes which results in a very short shelf life of approximately 3 days [10].

It was previously reported that generally fumigation is used for the management of cattle in the city of Kupang, and in the storage process it always uses food preservatives such as nitrite (KNO₂) [10]. Nitrite is one of the preservatives used in the process of preserving meat

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to obtain bright red color and prevent microbial growth [11–13]. As a preservative of meat, the amount of nitrite used in Australia is permitted up to 125 ppm; in USA, it is permitted between 200 and 500 ppm; and in Europe, it should not exceed 150 ppm [11]. In Indonesia, nitrite as a preservative is permitted for use by following the regulation of the Indonesia Drug and Food Supervisory Agency guidance (number 36 in 2013) regarding the maximum limit used in processed meat products. For poultry and mashed meat including se'i meat, the limit is 30 ppm, but in most cases, the nitrite used exceeds the usage limit stipulated by the Indonesia Drug and Food Supervisory Agency guidance [14]. Excessive consumption of nitrite causes health problems, because it binds to amines and amides present in meat proteins and forms nitrosamine derivatives that are toxic and can cause cancer [15–17].

Nitrite that enters the body through the food consumed can cause clinically significant health problems because it is absorbed in the lower digestive tract thereby causing the intestinal microbes to convert it into a more dangerous compound. Nitrite causes vasodilation in blood vessels, because it changes into nitric oxide (NO) which contains molecules that play a role in relaxing smooth muscles. Nitrite in the body will also bind to proteins forming *N*-nitroso [15]. Large amounts of *N*-nitroso in the body leads to the formation of reactive oxygen species (ROS), and as ROS increases, there will be an increase in the production of hydroxyl radicals (OH) that cause oxidative damage to protein molecules, such as DNA, and as a result, cell death occurs [15,18]. To prevent cell death, the cell will express heat shock proteins (HSPs), for example, wild-type p53 protein.

Wild-type p53 protein mutates and triggers the formation of cancer cells in the body [19] and plays a role in cells by preventing DNA damage, activating oncogenes and suppressing nutrient loss. Recent studies reported that wild-type p53 protein has been used as a marker for early detection of cancer (colon cancer) [20,21]. To our knowledge, until now early detection of colon cancer has not yet been carried out and there are no signs of an effort toward genetic development as an early detection tool. Various studies on the role of wild-type p53 protein in the growth of colon cancer have been widely reported [22–25]. However, the use of wild-type p53 as a means for early detection of colon cancer has not been widely reported. In this study, we report about the consumption effect of se'i beef meat (smoked beef which is traditionally processed from Kupang-Timor island, East Nusa Tenggara, Indonesia) that contains

nitrite compounds on the effect of wild-type p53 expression protein produces in mice colon cells. The hypothesis provides the effect of giving se'i meat on the expression of wild-type p53 cells in Balb/c mice as an indicator of carcinogenesis.

2 Materials and methods

2.1 Nitrite levels in the se'i

Nitrite in se'i meat was determined by the Griess method using sulfanilamide acid and *N*-1-naphthylethylene-diammonium dihydrochloride (NEDA) as a reactant [26]. The nitrite in the meat reacted with sulfanilamide acid in an acidic atmosphere and produced benzenediazonium ions. The formed benzenediazonium ions then coupled with NEDA and produced purple azo compounds. The formed azo compounds were then measured using UV-Vis spectroscopy at a wavelength of 520 nm.

2.2 Animals and experimental design

All test animals were approved by Veterinary Medicine, Airlangga University, Surabaya, Indonesia. Adult mice aged between 2.5 and 3 months were included in this study. Then the mice were acclimated for 7 days, which is done for the adjustment of experimental animals to the existing environmental conditions. Mice are kept in a ventilated room and individually grounded. Room temperature ranges from 28 to 32°C with a humidity of $56 \pm 5\%$. Food and drinks are given in the form of mice pellets, and the cage was cleaned every 2 days. Mice with an average body weight of 23.8 g were divided into the following four treatment groups: P1 (mice that have been given the standard food, drinking water and se'i meat that contains no nitrite), P2 (mice that have been given the standard food, drinking water and se'i meat containing nitrite from the modern industry), P3 (mice that have been given the standard food, drinking water and se'i meat containing nitrite from the home industry) and K (mice that have been given the standard food and drinking water). Randomization of two treatment groups (t) are group of mice that were fed se'i meat (P1, P2, P3) and group of control mice (K) was treated for 28 days (4 times the cell growth period). After that, on 28th day, the mice mole colon cells were examined to see the expression of wild-type p53 protein and Bcl-2 for sample and control.

2.3 Cell culture and reagents

Reagents used for immunohistochemical (IHC) staining were xylol, 95% absolute alcohol and 70% alcohol, buffered phosphate, H₂O₂ in methanol and citrate buffer. All reagents were purchased from Singapore Brands. Se'i meat taken from the modern and home industries in Kupang-Indonesia city, mice colon cells, anti-mouse p53 wild animals (Abcam), secondary anti-rat rabbit-based avidin–biotin detection system, 3,3'-Diaminobenzidine (DAB) peroxidase enzyme subtraction, core dyes (hematoxylin; MEyER) and Emerson oil were used in this study. Cell culture is carried out using the method of Piell et al. [18].

2.4 Determination of the amount of se'i beef to be provided

In the research conducted, the dose given for one mouse weighing 23.8 g was 8.840 mg. This dose is a conversion of consumption of se'i (g) by five people per day (2.857 g/day), which is then converted to mice weight as follows: from the results of the preliminary study, consumption per week = 100 g/5 people = 100 g/5 people/7 days = 2.857 kg/day (2.857 g/day for mice). Hence, the dose to be used in the study is 2.857 g. The meat conversion dose of 70 kg/human body weight (BW) for mice: $0.0026 \times 286 \text{ mg} = 0.007436 \text{ mg/kg BW mice}$; the dose for one mouse weighing 23.8 g = $0.74386 \times 23.8/20 = 0.00884 \text{ g}$ (8.840 mg), which was given using an oral sonde.

2.5 IHC examination of wild-type p53 protein

IHC examination of wild-type p53 protein was carried out using a method adapted from previous study [27]. Colon tissue was cut 4 μm with a microtome. This had previously been coated with poly-L-lysine and then placed on the object's glass, then deparaffinization was carried out, which is to remove paraffin in the tissue, by inserting these tissue incisions in succession into xylol for three times (for 2 min). At this point, put in a row 100% ethanol (three times) for 1 min, ethanol 95% (two times) for 1 min and ethanol 90%, 80% and 70% for 1 min each. Demineralization was carried out for about 5 min. Then, 3% hydrogen peroxide was used for 30 min to remove endogenous peroxidase. The next step is the

use of demineralized water washing and then neutralize with phosphate-buffered saline (PBS) for 2 min each. The sample was then put into 0.025% trypsin solution in PBS (pH 7.4) for 6 min at 37°C and washed with PBS three times (for 2 min). Furthermore, the sample was put into a primary antibody solution (p53 anti-mouse/rat) for 30 min and washed with PBS three times (for 2 min). After the process with PBS, the sample was put into a secondary antibody (rabbit anti-rat) for 30 min and then washing using PBS was continued two times (for 2 min). Furthermore, then HRP-conjugated streptavidin was added to the samples for 30 min, washed with PBS three times (for 2 min) and then put into the chromogen substrate for 5 min (DAB solution).

The samples were then washed again with PBS three times for 2 min, rinsed with demineralized water and put into Mayer's hematoxylin for 6 min and then washed again with demineralized water. The samples were analyzed using a light microscope (OLYMPUS DP40 microscope). After observation, IHC staining of the wild-type p53 protein was performed or crushed with wild-type p53 monoclonal antibody and analyzed and interpreted. Assessment of the expression of wild-type p53 was carried out quantitatively, by counting the number of cells expressing wild-type p53 in 10 visual fields with 400 \times magnification.

2.6 Statistical analysis

Statistical analysis of the data used in this study was carried out by the *F* test (one-way analysis of variance with $\alpha = 0.05$). Then, the differences that occur between groups were found by the post hoc test using least significant difference (LSD), whereas the relationship between wild-type p53 expression was found out by Pearson product-moment test.

3 Results and discussion

3.1 Nitrite levels in the se'i meat

A total of 125 meat samples were taken by accidental sampling techniques from Kupang city and then the nitrite levels were measured. Nitrite is a salt of weak acids and strong bases, in the form of crystals that are pale yellow and easily soluble in water. It is also a

Table 1: Results of analysis of nitrite content in meat

Company	Company code	Nitrite content (mg/kg)
Modern industry	DA	34.68 and 35.10*
	SR 1	26.09
	SR 2	27.68
	Average	29.48
Home industry	Psr 1	22.28
	Psr 2	110.19 and 76.54*
	Psr 3	37.42
	Average	56.63

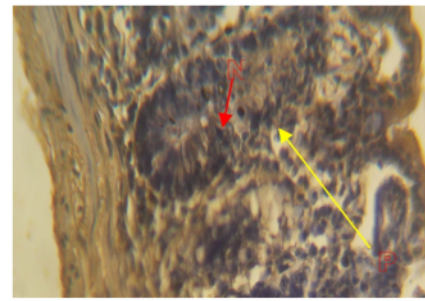
*Tested twice for nitrite in meat.

highly reactive compound that acts as a reducing agent and an oxidizer, as well as a nitrosylating agent (forming NO) [15]. It can be used in the curing process and will form NO_2^- and HNO_2 neutral nitrous acids. Small amounts of HNO_2 acid can form highly reactive nitrosating species [13,28]. In a slightly acidic condition, it will react with the meat component so that nitric oxide (NO) and nitric acid (HNO_3) are formed. Meat pigments are formed when myoglobin is directly in contact with NO so that brightly colored nitric oxide myoglobin (nitrosomyoglobin) is formed [29]. Heating during the treatment process causes nitrosomyoglobin to turn into a stable and pink nitrosylheme-chrome [30]. The results of the analysis of nitrite content in meat of six samples, with three samples originating from the home industry and three samples from the modern meat processing industry, are shown in Table 1.

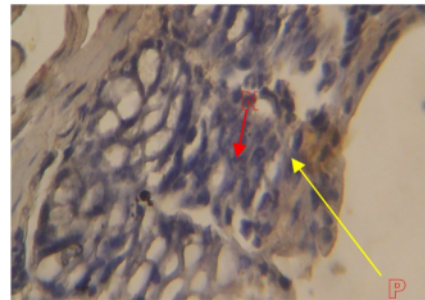
In Table 1, it can be seen that all samples of se'i meat originating from the home industry groups contain nitrite exceeding the threshold, whereas the samples from modern industries exceed the threshold but some are below the threshold of 30 mg/kg according to regulations of The Republic of Indonesia Drug and Food Supervisory Agency. In this research, meat from the home industry groups contains 110.19 mg nitrite, whereas that from the modern industry group contains 34.68 mg nitrite.

3.2 IHC examination results of expression of p53 protein in colon cells of Balb/c mice

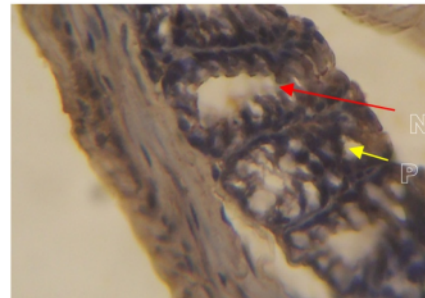
Immunohistochemistry (IHC) was chosen to find out the effect of giving meat on wild-type p53 protein expression in mice because IHC is a technique that can be used to determine the presence and level of specific cellular proteins sample. Examination of wild-type p53 protein expression in mice colon cells using the OLYMPUS DP40 microscope showed that positive expression of the



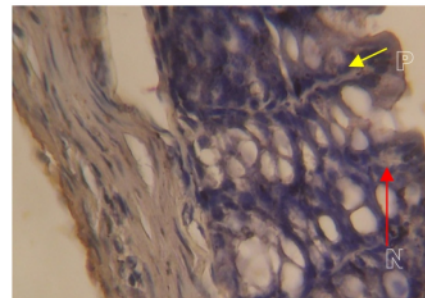
(a)



(b)



(c)



(d)

Figure 1: Expression of wild-type p53 protein in se'i: (a) home industry, (b) modern industry, (c) without nitrite and (d) control (note: positive [P sign] and negative [N sign] given meat se'i).

wild-type p53 protein was characterized by the presence of brown granules in colon cells (Figure 1).

Based on the test results of wild-type p53 expression with IHC staining in mice colon tissue with feeding treatment with meat that originated from home industry, modern industry, meat without nitrites and controls can be seen that cells expressing wild-type p53 are characterized by brown cytoplasm (P sign) while those with no brown staining on IHC staining of colon cells means that no wild-type p53 expression (N sign). The data from this study are supported by the results of descriptive analysis that show the average number (mean \pm standard deviation) of cells expressing wild-type p53 protein. The results of IHC staining of 36 male mice samples of Balb/c strain to see the expression of wild-type p53 protein are shown in Table 2 and Figure 2.

In Table 2 and Figure 2, it can be seen that the highest wild-type p53 expression value is from the group containing nitrites originating from home industries with an average of 142 and the lowest in the control group with an average of 78.11. The normality test uses the Shapiro–Wilk test for all treatment groups (from home industries, modern industries, without nitrites and controls), and normal data distribution is obtained.

Table 2: Descriptive analysis of expressions of wild-type p53 protein

Groups	N	Minimum	Maximum	Mean	SD
Se'i home industry	9	116	190	142	22.48
Se'i modern industry	9	84	171	126.22	24.14
Se'i without nitrite	9	85	140	106.55	17.27
Control	9	46	105	78.11	20.02

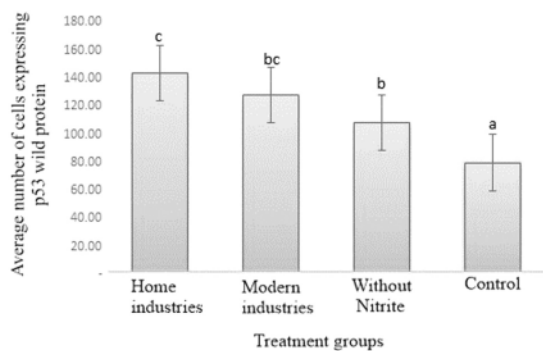


Figure 2: Histogram of the treatment group versus the average number of cells expressing the wild-type p53 protein (note: the same ab superscript shows no differences between groups [based on the LSD test]).

However, the results of different tests between groups of wild-type p53 expression in colonic cells of Balb/c mice (Figure 2) show that there are significant differences between the control group and the treatment group.

These results indicate that se'i meat has the ability to express wild-type p53 protein in all treatment groups. Increased wild-type p53 expression is influenced by adaptation factors whose function inhibits the cell division cycle or balances proliferation and apoptosis (references). The average nitrite content in se'i meat given to mice for 28 days is still normal, which causes wild-type p53 expression which is offset by an increase in Bcl-2 expression in mice colon cells so as not to cause excessive cell cycles which can cause malignancy. It has The results indicate that increased expression of wild-type p53 protein in mice colon due to feeding of se'i meat, causes the activation of apoptosis through extrinsic pathways, which include activation of Death Receptors (DR) such as DR5 expression. DR5 will then activate caspase-8. Caspase-8 will then catalyze a series of proteolytic processes that produce typical biochemical and morphological changes that can suppress or simultaneous repression of Bcl-2 [31].

4 Conclusion

The results indicated that there is a difference in the expression of wild-type p53 in P1 (mice that have been given the standard food, drinking water and se'i meat that contain no nitrite), P2 (mice that have been given the standard food, drinking water and se'i containing nitrite from modern industry), P3 (mice that have been given the standard food, drinking water and se'i meat containing nitrite which come from the home industry) and K (mice that have been given the standard food and drinking water) groups. In the near future, it is hoped that further research can be carried out on the effects of se'i meat containing nitrite on the expression of p53 protein in other organs.

Conflict of interest: The authors declare no conflict of interest.

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