The profile of spermatogenic cells and superoxide dismutase in the testis of rats under boiled grobogan tempe and soybean flour treatment

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Abstract. Tempe is a traditional food that is well-known in Indonesia, made by fermentation of boiled soybeans using Rhizopus sp. Isoflavones are the bioactive compound in tempe. The aim of this research was to analyze the number of spermatogenic cells, the number of Leydig cells, and antioxidant Copper,Zinc-Superoxide Dismutase (Cu,Zn-SOD) content in the testicular tissues of rats treated with boiled Grobogan tempe and soybean flour. This research used 15 Sprague-Dawley rats that were divided into five groups: casein treated group, boiled Grobogan tempe flour 10% treated group, boiled Grobogan tempe flour 20% treated group, boiled Grobogan soybean flour 10% treated group, and boiled Grobogan soybean flour 20% treated group. The treatment was given for 90 days (European Food Safety Authority - subchronic test). The testicular tissues were subjected to standard method tissue processing using paraffin and staining using hematoxylin-eosin and an immunohistochemical technique for the content of Cu,Zn-SOD. The results showed that boiled Grobogan tempe and soybean flour increased the number of spermatogenic cells, the number of Leydig cells, and Cu,Zn-SOD antioxidant content in the testicular tissues of experimental rats. Treatment of boiled Grobogan tempe flour 20% gave the best result in increasing Cu,Zn-SOD antioxidant in the testicular tissues.

1. Introduction
Tempe is a traditional food from Indonesia which is produced through a fermentation process using the mold Rhizopus sp., forming a dense and compact mass. In general, tempe in Indonesia is produced using soybeans. Approximately 50% or 1.3 tons of the national total soybean supply is absorbed by tempe makers [1]. This indicates that tempe is a popular Indonesian food.

A number of tempe’s advantages compared to soybeans are that it has a better protein, carbohydrate, and fat digestibility, a higher content of some vitamins, a better mineral bioavailability due to the elimination of anti-nutrient materials, and the presence of bioactive components which are not found in soybeans [2]. These bioactive components have antioxidant and antidiarrheal activities and can prevent degenerative diseases [3,4]. Astuti et al. [3] reported that tempe has many benefits for humans, for example reducing flatulence and diarrhea, inhibiting the formation of cholesterol in the
liver, preventing oxidation of low-density lipoprotein (LDL), reducing the total cholesterol, increasing the antioxidant SOD and reducing cancer risks.

Besides having good quality protein, tempe also contains isoflavones which could scavenge free radicals [5,6]. Sikka [7] reported that natural foodstuffs that contain antioxidants act as free radical scavengers which could suppress the oxidation process, lipid peroxidation, and spermatozoa damage, prevent oxidative stress conditions, and increase the antioxidant status. In addition, tempe also has nutritive values equal to other protein sources (beef, cow’s milk, chicken eggs) [8].

Not much is known about the effect of tempe on the superoxide dismutase (SOD) antioxidant content in testes in supporting the spermatogenesis and its effect in relation to feminism. Therefore, there needs to be an analysis of the effect of feeding tempe flour and boiled soybeans on the spermatogenic cell profile, Leydig cells, and SOD antioxidant content in the testes through immunohistochemical detection using male rats as the experimental animal. In this study, the tempe used was made from local Grobogan soybeans, a new soybean variety which is cultivated in large amounts in Grobogan and has been identified as having good quality [9]. This study aimed to analyze the histological profile of spermatogenic cells, Leydig cells, and the superoxide dismutase (SOD) antioxidant content in the testes of rats fed Grobogan tempe flour and boiled Grobogan soybeans.

2. Materials and methods
2.1. Materials and equipments
The materials used in this study were Sprague-Dawley male rats, casein, Grobogan tempe flour, Grobogan soybean flour, alcohol, xylol, paraffin, entellan®, ketamine, xylazine, hematoxylin-eosin, phosphate buffer saline (PBS), H2O2, methanol, normal serum, a background sniper, Copper primary monoclonal antibody, Zinc-Superoxide Dismutase (Cu,Zn-SOD, Sigma S2147), diaminobenzidine (DAB), Trekkie avidin, and Trekkie universal link.

This study used the following equipment: tweezers, labels, gloves, sample vials, Petri dishes, beaker glasses, graduated cylinders, filter paper, tissue basket, tissue embedding console, object glasses, microscope slide covers, a set of staining equipment, digital scales, micropipettes, an incubator, a microtome, and a photomicroscope.

2.2. Treatments, sampling and testicular tissue processing
The experimental rats were divided into five treatment groups fed feed with dietary protein from casein (A), Grobogan tempe flour 10% (B), Grobogan tempe flour 20% (C), Grobogan soybean flour 10% (D) and Grobogan soybean flour 20% (E). The flours had been formulated according to the dietary standards of the Association of Official Analytical Chemists [10]. The flours were fed to the rats for 90 days following the guidelines of the European Food Safety Authority [11], guidelines for food/feed toxicity studies with repeated dosages for 90 days (subchronical testing) on experimental animals with a few modifications.

Testis sampling was conducted at the end of the treatment period by anesthetizing the rats using a combination of ketamine 70mg/kg BW and xylazine 20mg/kg BW injected intraperitoneally. After the rats were anesthetized, a median laparotomy was conducted and the testes were collected then washed in 0.9% physiological NaCl solution and placed in Bouin’s fixative solution. The testicular tissue processing was done using the standard method of embedding with paraffin. Then the tissue block was sliced using a microtome and was stained using hematoxylin-eosin stain [12], and immunohistochemical staining using superoxide dismutase monoclonal antibody [13, 14].

2.3. Data analysis
Data analysis was conducted both quantitatively and qualitatively. Quantitative observations were made by counting the number of spermatogenic cells: spermatogonium cells, primary spermatocytes, early spermatids and late spermatids in the testis in the stage VIII wave of the seminiferous tubule [15]. Qualitative observations were made by observing the morphology of spermatogenic cells and the content of the antioxidant Cu,Zn-SOD in the testis.
The Cu,Zn-SOD content in testicular tissue was visualized by a brown color in the nucleus and cytoplasm. Observations were made based on the intensity of the color formed in the nucleus and cytoplasm; the darker the brown means the more Cu,Zn-SOD contained. Cells that do not contain Cu,Zn-SOD would appear blue (hematoxylin) in the nucleus due to the counterstain. The estimation of Cu,Zn-SOD content was classified into three color intensities for positive reactions and one color for negative reactions. A positive reaction to Cu,Zn-SOD in testicular tissue is marked by a dark brown color covering the entire nucleus or a very strong positive (+++), a dark brown nearly covering the entire nucleus or a strong positive (++), a light brown covering part of the nucleus or a medium positive (++), and brown streaks in the nucleus or a weak positive (+). A negative reaction (-) is marked by the entire nucleus stained blue due to the counterstain (hematoxylin). The cell count was done at a 10x magnification in five random fields in each tissue slide [13] using the McMaster Biophotonics Image J software program then tested with the analysis of variance (ANOVA) using the IBM SPSS release 22 software program. If the test resulted in a significant difference (P<0.05), it was continued with Duncan’s test. Qualitative data were presented descriptively in the form of pictures.

3. Results and discussion

3.1. The Number of Spermatogenic Cells in the Treatment Rats Testes

The photomicrograph of rats testicular tissues stained using hematoxylin eosin was showed in Figure 1. The number of spermatogenic cells per seminiferous tubule in the rat testicular tissue in various treatment groups are presented in Table 1. The number of spermatogenic cells was observed in the stage VIII wave of the seminiferous tubule [15], which has a complete cell composition in the process of spermatogenesis.

Table 1. The number of spermatogenic cells per seminiferous tubule in the rat testicular tissue in various treatment groups.

| Treatment | Spermato Gonia | Primary Spermatocytes | Early Spermatids | Late Spermatids | Number of Spermatogenic Cells |
|-----------|----------------|-----------------------|-----------------|----------------|-----------------------------|
| I         | 45.20 ± 5.53a  | 61.60 ± 7.84a         | 144.07 ± 26.31a | 100.87 ± 24.40a | 351.73 ± 33.68a            |
| J         | 51.13 ± 5.82bc | 59.27 ± 4.71a         | 162.87 ± 17.37bc| 107.53 ± 17.94b | 380.80 ± 25.73b            |
| K         | 52.73 ± 3.59b  | 67.27 ± 7.43b         | 174.47 ± 23.08c | 118.13 ± 12.21bc | 412.60 ± 27.06c            |
| L         | 47.93 ± 5.19ab | 62.40 ± 6.98ab        | 152.87 ± 13.24ab| 122.20 ± 14.55c | 385.40 ± 18.20b            |
| M         | 51.00 ± 6.58bc | 64.00 ± 6.36ab        | 155.53 ± 14.70ab| 123.73 ± 20.37c | 394.27 ± 34.03bc           |

I = control (standard casein feed); J = boiled Grobogan tempe flour 10%; K = boiled Grobogan tempe flour 20%; L = boiled Grobogan soybean flour 10%; M = boiled Grobogan soybean flour 20%. Different letters in the same column mark a significant difference (P<0.05)

The group of rats fed boiled Grobogan tempe flour 10% (J) had a significantly higher number of spermatogenic cells (P<0.05) than the group of rats fed casein (table 1). Similar results were demonstrated by rats fed boiled Grobogan tempe flour 20% (K), which had a significantly higher number of spermatogenic cells (P<0.05) than the group of rats fed casein (table 1). However, the group of rats fed boiled Grobogan tempe flour 20% (K) had a significantly higher number of spermatogenic cells (P<0.05) than rats fed boiled Grobogan tempe flour 10% (J) (table 1). The high number of spermatogenic cells is believed to be associated with the amount of protein and isoflavones in boiled Grobogan tempe flour 20% which is higher than those in boiled Grobogan tempe flour 10%, which improved the health of the spermatogenic cells, resulting in a higher spermatogenic cell count in the experimental rats.

The number of spermatogenic cells in rats fed boiled Grobogan soybean flour 10% (L) did not demonstrate a significant difference (P>0.05) from rats fed boiled Grobogan soybean flour 20% (M) (table 1). However, both had a significantly higher number of spermatogenic cells (P<0.05) than rats fed casein (table 1). The difference in Grobogan soybean flour concentration did not have a significant
effect on the increase in spermatogenic cell numbers. This demonstrated that boiled Grobogan soybean flour 10% and 20% had similar qualities in increasing the number of spermatogenic cells.

Figure 1. Fotomicrograph of seminiferous tubules in rats testicular tissues stained with heamatoxylin eosin. I = control (standard casein feed); J= boiled Grobogan tempe flour 10%; K= boiled Grobogan tempe flour 20%; L= boiled Grobogan soybean flour 10%; M= boiled Grobogan soybean flour 20%. Scale = 50 μm.

When seen from the protein content percentage of Grobogan tempe and boiled soybeans, group K (boiled Grobogan tempe flour 20%) had a significantly higher number of spermatogenic cells (P<0.05) than groups J and L (Grobogan tempe flour and boiled soybean 10%). However, group M resulted in a number of spermatogenic cells that was not significantly different (P<0.05) from groups J, K, and L (table 1). In general, it can be seen that the four groups of rats fed Grobogan tempe flour and boiled soybean flour, both 10% and 20% (J, K, L, and M) resulted in spermatogenic cell numbers that were significantly higher (P<0.05) than the group of rats fed casein (table 1). This was supported by the numbers of spermatogonia in groups J, K, and M which were significantly higher (P<0.05) than that of group I (table 1). Even though the number of spermatogonia in group I was not significantly different (P<0.05) from that of group L and the number of spermatogonia in group L was not significantly different (P<0.05) from those of groups J and M, group I was significantly lower than groups J and M (table 1). The increase in spermatogenic cell numbers was believed to be associated with the presence of a bioactive substance in tempe and soybean, isoflavone. Astuti et al. (2008) also reported that the administration of low-fat soybean flour which is rich in isoflavone could increase the number of spermatogenic cells in the seminiferous tubules of rat testes.

The higher numbers of spermatogenic cells in rats fed Grobogan tempe flour and soybeans than that of rats fed casein is believed to be associated with the quality of protein found in tempe and soybeans. According to Astawan et al. [5] tempe flour and boiled soybeans have better quality protein than casein. The good protein quality found in tempe flour and soybean will help regeneration, growth, and maintenance of cell, tissue, and organ structures [16]. Therefore, the protein found in tempe and soybean would have a major role in the increased number of spermatogenic cells. This demonstrates that the administration of Grobogan tempe flour and boiled soybeans (10% and 20% concentration) did not reveal any indicators of feminism in the experimental male rats.
3.2. The number of Leydig cells in the testes of the treatment rats

The number of Leydig cells per seminiferous tubule in the rat testicular tissue for various treatment groups are presented in table 2. The group of rats fed boiled Grobogan tempe flour 10% (J) had a significantly higher number of Leydig cells (P<0.05) than the group fed casein (I) (table 2). The number of Leydig cells in rats fed boiled Grobogan tempe flour 20% (K) was also significantly higher (P<0.05) than that in rats fed casein (table 2). However, between the two treatments (J and K), group K had a higher number of Leydig cells than group J (table 2). The increased number of Leydig cells also affected the increase in the spermatogenic cell count. This is supported by the data in table 1 which shows that the number of spermatogenic cells in the rats in groups J and K were significantly higher (P<0.05) than that of the group fed casein.

Rats fed boiled Grobogan soybean flour 10% (L) had a significantly higher number of Leydig cells (P<0.05) than rats fed casein (table 2). On the other hand, the group of rats fed boiled Grobogan soybean flour 20% (M) had a significantly higher number of Leydig cells (P<0.05) than the group of rats fed boiled Grobogan soybean flour 10% (L) (table 2). Based on the concentration of Grobogan tempe flour and soybeans, the number of Leydig cells in rats fed the two types of flour with a concentration of 20% (K and M) had significantly higher number of Leydig cells (P<0.05) than rats fed the two types of flour with a concentration of 10% (J and L) (table 2).

Table 2. The number of Leydig cells per interstitial seminiferous tubule in the rats’ testicular tissue for various treatment groups.

| Group | Number of Leydig cells |
|-------|------------------------|
| I     | 60.47 ± 4.64<sup>a</sup> |
| J     | 71.07 ± 8.18<sup>b</sup> |
| K     | 80.87 ± 7.42<sup>c</sup> |
| L     | 69.80 ± 8.06<sup>b</sup> |
| M     | 76.80 ± 6.72<sup>c</sup> |

I = standard casein feed; J= boiled Grobogan tempe flour 10%; K= boiled Grobogan tempe flour 20%; L= boiled Grobogan soybean flour 10%; M= boiled Grobogan soybean flour 20%. Different letters in the same column mark a significant difference (P<0.05).

The group of rats fed Grobogan flour and boiled soybeans 20% (K and M) demonstrated the highest number of Leydig cells, having the strongest influence on the development of spermatogenic cells in the seminiferous tubule of the testes. This is assumed to be due to the high testosterone level produced by the Leydig cells which support the process of spermatogenesis in the seminiferous tubules of the testes (table 1). Astuti et al. [17] reported that the increased number of Leydig cells indicated an increase in testosterone levels that would increase the number of spermatogenic cells and could improve the process of spermatogenesis. The increased number of spermatogenic cells and Leydig cells, which also means that the hormone testosterone is increased, could be used as information that the consumption of Grobogan tempe flour and boiled soybean at concentrations of 10% and 20% did not cause any indicators of feminism in the experimental rats.

3.3. The Cu,Zn-SOD Content Profile in Spermatogenic Cells of the Model Rats’ Testes

The Cu,Zn-SOD content was observed in the stage VIII wave of the seminiferous tubule [15], (figure 2). This was very helpful in analyzing the Cu,Zn-SOD content because during the stage VIII wave the seminiferous tubule has a complete cell composition in the process of spermatogenesis. The complete composition of cell types in the seminiferous tubule would provide specific information about the antioxidant Cu,Zn-SOD content profile both in every type of cell and the general profile in each seminiferous tubule.
Figure 2. Fotomicrograph of immunolocalization of antioxidant SOD in the seminiferous tubules in testicular tissues treated rats. I = control (standard casein feed); J= boiled Grobogan tempe flour 10%; K= boiled Grobogan tempe flour 20%; L= boiled Grobogan soybean flour 10%; M= boiled Grobogan soybean flour 20%. Scale = 50 μm.

The group of rats fed boiled Grobogan tempe flour 10% (J) revealed a higher antioxidant Cu,Zn-SOD content than the group of rats fed casein. On the other hand, group J had a lower antioxidant Cu,Zn-SOD content than the group fed boiled Grobogan tempe flour 20% (K). This was supported by the number of primary spermatocytes in group K which gave a very strong positive reaction which was significantly higher (P<0.05) than group L (table 3). Pertaining to early spermatid and late spermatid cells, group K also had a significantly higher number of cells that had a strong positive reaction (P>0.05) than group L (table 3). The administration of boiled Grobogan tempe flour 20% was better in increasing the antioxidant Cu,Zn-SOD content in the experimental rats’ testes compared to boiled Grobogan tempe flour 10% or casein. The high antioxidant Cu,Zn-SOD content in testicular tissue would improve the oxidative defense mechanism (the body’s antioxidant status), enabling it to increase the number of spermatogenic cells (table 1) and Leydig cells (table 2) in group K.

The antioxidant content in the testes of rats in group L was similar to that of group M. This was supported by the number of spermatogonia cells and primary spermatocytes in group L which gave a very strong positive which was not significantly different (P>0.05) from that of group M (table 3). Group L also had a number of primary spermatocytes and late spermatids which gave an intermediate/weak positive reaction and early spermatid cells which gave a strong positive which were similar to or not significantly different (P>0.05) from those of group M (table 3). Based on the antioxidant content in the testicular tissue of rats in groups L and M, Grobogan soybean flour 10% and 20% had similar qualities. However, the two had a better quality than casein. This finding was supported by the numbers of spermatogonia cell nuclei that gave very strong positive reactions in groups L and M which were significantly higher (P<0.05) than those in rats fed casein (table 3).
| Group            | Cu,Zn-SOD content level | I               | J               | K               | L               | M               |
|-----------------|-------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Spermatogonia   | +++                     | 14.27 ± 4.45a   | 20.40 ± 5.69bc  | 23.60 ± 5.28b   | 19.53 ± 5.37b   | 19.53 ± 3.62b   |
|                 | +++                     | 14.67 ± 4.98    | 12.47 ± 4.45    | 12.00 ± 5.87    | 12.80 ± 5.10    | 14.60 ± 5.80    |
|                 | ++/+                    | 15.07 ± 4.15    | 16.07 ± 6.28    | 15.87 ± 4.78    | 15.33 ± 3.29    | 16.53 ± 4.39    |
|                 | -                       | 0.20 ± 0.77     | 0.13 ± 0.35     | 0.47 ± 1.13     | 0.00 ± 0.00     | 0.20 ± 0.41     |
| Primary Spermatocytes | +++                   | 9.73 ± 5.59a    | 11.33 ± 4.53a   | 15.80 ± 4.33b   | 12.07 ± 4.76ab  | 11.07 ± 6.52a   |
|                 | +++                     | 27.13 ± 7.00    | 26.67 ± 8.05    | 31.87 ± 11.44   | 23.73 ± 7.97    | 24.67 ± 7.61    |
|                 | ++/+                    | 24.67 ± 7.64abc | 20.40 ± 8.38ab  | 19.13 ± 5.87a   | 25.60 ± 9.72bc  | 28.07 ± 6.72c   |
|                 | -                       | 0.07 ± 0.26     | 0.00 ± 0.00     | 0.00 ± 0.00     | 0.00 ± 0.00     | 0.00 ± 0.00     |
| Early Spermatids | +++                    | 0.00 ± 0.00     | 0.00 ± 0.00     | 0.00 ± 0.00     | 0.13 ± 0.52     | 0.00 ± 0.00     |
|                 | +++                     | 1.40 ± 1.64a    | 12.47 ± 12.99b  | 23.60 ± 22.39c  | 3.13 ± 5.03ab   | 6.80 ± 7.00ab   |
|                 | ++/+                    | 141.87 ± 26.50  | 141.47 ± 29.63  | 144.80 ± 36.17  | 149.60 ± 16.37  | 143.53 ± 23.61  |
|                 | -                       | 0.80 ± 1.66     | 0.27 ± 0.80     | 1.07 ± 2.63     | 1.67 ± 5.92     | 2.33 ± 5.15     |
| Late Spermatids  | ++++                   | 0.00 ± 0.00     | 1.87 ± 2.97     | 2.07 ± 3.73     | 0.33 ± 1.29     | 0.67 ± 1.84     |
|                 | +++                     | 2.07 ± 3.83a    | 4.60 ± 5.21bc   | 7.80 ± 4.92c    | 6.13 ± 6.21c    | 5.73 ± 4.45ab   |
|                 | ++/+                    | 0.73 ± 1.49a    | 3.00 ± 4.09ab   | 5.80 ± 6.19abc  | 7.73 ± 7.11c    | 4.00 ± 3.42abc  |
|                 | -                       | 98.07 ± 23.52   | 92.87 ± 15.75   | 99.00 ± 14.87   | 108.00 ± 15.61  | 108.33 ± 18.43  |

I = standard casein feed; J = boiled Grobogan tempe flour 10%; K = boiled Grobogan tempe flour 20%; L = boiled Grobogan soybean flour 10%; M = boiled Grobogan soybean flour 20%. Different letters in the same row mark a significant difference (P<0.05)
The profiles of the antioxidant Cu,Zn-SOD content in the group of rats fed boiled Grobogan tempe flour 10%, boiled Grobogan soybean flour 10% and 20% (J, L, and M) were similar. However, the group of rats fed boiled Grobogan tempe flour 20% (K) had a higher antioxidant Cu,Zn-SOD content than groups I, J, L, and M. This finding was supported by the number of early spermatid cells that gave a strong positive was significantly the highest (P<0.01) when compared to those in groups I, J, L, and M (table 3). It is assumed that in the group of rats fed boiled Grobogan tempe flour 20% the number of free radicals existing could be neutralized by the high antioxidant content.

Isoflavones contained in tempe have an important role as an antioxidant. According to Astawan [18], isoflavones in soybean fermentation products (among them tempe) are in a free form (aglycone). The free form of isoflavones is more readily absorbed by the small intestines [19], resulting in a larger amount of antioxidants being absorbed by the body. This would decrease the use of endogenous antioxidants and increase the body’s antioxidant status and maintain a high content of the antioxidant Cu,Zn-SOD [20]. Tempe as food containing isoflavone as an antioxidant is believed to play a role in protecting the spermatogenic cells in the testes with its ability as a free-radical scavenger. Astuti [21] also reported that isoflavones from soybean flour could increase the content of the antioxidant Cu,Zn-SOD in spermatogenic cells and could protect those cells and act as a free radical scavenger.

4. Conclusion

The administration of Grobogan tempe flour and boiled Grobogan soybean could increase the number of spermatogenic cells, Leydig cells and the antioxidant Cu,Zn-SOD content in rat testicular tissue and was better than the administration of standard casein feed. Grobogan tempe flour 10%, and boiled Grobogan soybean flour 10% and 20% had similar qualities in increasing the antioxidant Cu,Zn-SOD content in rat testes. The administration of Grobogan tempe flour and boiled Grobogan soybean 20% resulted in a higher number of spermatogenic cells and Leydig cells compared to the administration of Grobogan tempe flour and Grobogan soybean 10% and casein. The best treatment was the administration of Grobogan tempe flour 20% which resulted in high spermatogenic cells and Leydig cells numbers and had the highest antioxidant Cu,Zn-SOD content compared to the other treatments.

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