The Comparative Effect of Fasting with and without Caloric Restriction in Rat on Oxidative Stress Parameters

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Introduction: Fasting, like Islamic Ramadan Fasting, has been associated with health benefits. Islamic Ramadan fasting, a form of caloric restriction (CR) or alternate day fasting that. Studies suggest a comparable effect of ADF and caloric restriction. Despite the fact that fasting can be considered as a form of dietary restriction, fasters tend to have difficulty to reduce their food intake during non-fasting period by overeating leading to the excessive calorie intake. To compare the effect of fasting with and without caloric restriction in Sprague Dawley rats.

Methods: The rats were assigned to one of three groups: ADF with 70 % calorie intake (30% CR), ADF with 100 % calorie intake (0% CR), and ADF with 140 % calorie intake (excessive calorie intake) and AL (fed ad libitum). All groups were subjected to 6 hour fasting per day (9 a.m. until 3 p.m.) or 15 days. The plasma sample was taken for MDA level assessment Urinary 8-oxodG levels were determined by using ELISA.

Results: ADF with 30% calorie restriction (F70) group had the lowest MDA level. Measurement of 8-oxodG level showed that group F70 had the highest production of 8-oxodG. There was an inverse relationship between MDA level and 8-oxodG level meaning the lower MDA level, the lower 8-oxodG levels were produced. 

Conclusion: ADF fasting with 30% caloric restriction reduce the MDA level but increase 8-oxodG levels. This study suggest the beneficial effect of fasting requires decrease in overall caloric intake.
that mice subjected ADF consumed relatively the same amount of food in 48-h period as did that male mice in those fed AL. These excessive calorie intake has been shown to increase in blood glucose level as well as corresponding production of ROS and inflammatory cytokines (7), thus increasing degenerative diseases risks.

Malondyaldehyde (MDA) and 8-oxo-7, 8-dihydro-2’deoxyguanosine (8-oxodG) are oxidative stress indicators generated due to the interaction between free radicals with cell membrane and DNA core. MDA is generated as the result of lipid peroxidation when hydroxyl (OH) free radical disentangles 1 hydrogen atom from polyunsaturated fatty acid (PUFA) at the phospholipid layer of cell membrane (8). In addition, 8-oxodG is generated due to the interaction of hydroxyl (OH) free radical with purine bases of DNA core or mitochondria (9). Therefore, the decrease production of MDA and 8-oxodG will lead to the decrease in the degenerative diseases risks.

A study suggests the need to study the benefit of ADF while also considering the potential risks of increased calorie intake commonly occur during non-fasting periods (10). To gain insight into the health benefit effect of fasting, this study compared the effect of fasting with and without caloric restriction in rats.

**Material and methods**

**Animal and Food Intake**

Twenty four male 250 to 300 g Sprague Dawley were obtained and housed in Centre for Food and Nutrition of Gajah Mada University laboratory. The rats were assigned to one of three groups: ADF with 70 % calorie intake (30% CR), ADF with 100 % calorie intake (0% CR), and ADF with 140 % calorie intake (excessive calorie intake) and AL, fed ad libitum. All groups were subjected to 6 hour fasting per day (9 a.m. until 3 p.m.) or 15 days. The amount of calories needed for an adult human (70kg) is about 2000 kcal/day. Hence, the amount of calories needed for a 200 gram-rat is about 36 kcal/day. All groups were subjected to 6 hour fasting per day (9 a.m. until 3 p.m.) or 15 days. The amount of calories needed for an adult human (70kg) is about 2000 kcal/day.

Meanwhile, pellets and water were provided ad libitum for rats in group 4. At days 16, MDA level in plasma was measured. All procedures were approved by Bioethic committee of Medical Faculty, Sultan Agung Islamic University.

**Table 1. AIN-93M composition (31)**

<table>
<thead>
<tr>
<th>INGREDIENT</th>
<th>(g/kg diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch (&gt;85% protein)</td>
<td>465.992</td>
</tr>
<tr>
<td>Dextrinized cornstarch (90-94% tetrasaccharides)</td>
<td>140.000</td>
</tr>
<tr>
<td>Sucrose</td>
<td>155.000</td>
</tr>
<tr>
<td>Soybean oil (no additives)</td>
<td>106.000</td>
</tr>
<tr>
<td>Fiber</td>
<td>40.000</td>
</tr>
<tr>
<td>Mineral mix (AIN-93G-MX)</td>
<td>35.000</td>
</tr>
<tr>
<td>Vitamin mix (AIN-93-VX)</td>
<td>10.000</td>
</tr>
<tr>
<td>L-cystine</td>
<td>1.800</td>
</tr>
<tr>
<td>Choline bitartrate (41.1% choline)</td>
<td>2.500</td>
</tr>
<tr>
<td>Tert-Butylhydroquinone (TBHQ), mg</td>
<td>8.0</td>
</tr>
</tbody>
</table>

**MDA Assay**

The MDA assay was conducted by the method of Thiobarbituric acid reactive substances (TBARS) assay reported by Moselhy (11). MDA analysis is a method based on the reaction of thiobarbituric acid with some lipid peroxidation products in an acidic environment at an increased temperature. The product forms a pink colour enabling its spectrophotometric assay. The procedure starts with the addition of 1.5 mL of 20% acetic acid, 1.5 mL of 0.8% thiobarbituric acid, 200 µL of 8.1% sodium dodecil sulphate, and 700 µL of distilled water to a 100 µL sample. After being heated in boiling water for 60 min and 1 mL of distilled water and 5 mL of butanol/pyridine (14/1: v/v) were added to the samples. The samples were then centrifuged before the measurement of supernatant absorbance.

**Urinary 8-oxodG determination**

Urinary 8-oxodG levels in urine were determined by using ELISA assay described previously (12). Briefly, after collected and agitation, the volume of the sample was measured. 2 £ 1ml of the homogenized urine were kept at 280°C. To 1 ml of urine, 100ml of 3mol/l Tris-EDTA solution pH 8.6 were added and vortex mixed for 30s. After that, the solution was applied to Bond Elute C18 (OH) SPE (3ml) column that had been prepared with 3ml methanol and 3ml water. The column was washed with 3ml water followed by 3ml 2.5% acetonitrile and 1.5% methanol in 10mmol/l borate pH 7.9. The sample was eluted with 3ml of the same buffer and applied to a Bond Elute strong cation exchange column (3ml) prepared...
with 3ml methanol and 3ml borate buffer pH 7.9. The 8-oxo-dG was eluted with 2ml of acetonitrile/methanol buffer in borate and then adjusted to pH 6.0 with 1mol/l HCl. About 4ml of 50:50 dichloromethane was added and vortex mixed for 30s. The sample was then centrifuged for 10 min at 3500 rpm, the upper aqueous layer aspirated off and 3ml of organic layer evaporated to dryness under nitrogen at 50°C. Finally, the sample was reconstituted in 1ml HPLC running buffer without acetonitrile and 50ml injected into HPLC column. The 8-oxo-dG values were expressed as the ratio to creatinine urine concentration given in mmol/ml.

Statistics
Results were calculated as means ± standard error of the mean (SEM). Statistical significance was determined by One-Way ANOVA followed by post hoc test. A value of P<0.05 was considered significant.

Results
As shown in table 2, measurement of MDA plasma by using showed that ADF with 30% calorie restriction (F70) group had the lowest MDA level (0.770 nmol/mL) followed by ADF with 0% (F100) group (1.298 nmol/mL), AL (calories-ad libitum) group (3.924 nmol/mL). Interestingly, although all rats in group ADF with excessive calorie (F140) group were subjected to fasting, excessive calorie consumption (140% total calories intake), they had higher MDA level compared to the other groups with less total calorie intake. Measurement of 8-oxo-dG level, interestingly, showed that group F70 had the highest production of 8-oxo-dG (5.040 ng/mL), followed by F100 group (3.260 ng/mL), AL (2.660 ng/mL) group, and F140 group (2.580 ng/mL). The result of MDA and 8-oxo-dG levels were then analyzed with one-way ANOVA. For parametric test, the data were normally distributed (Shapiro-Wilk, P<0.05) and equal (Levene test, P<0.05). The statistical analysis using one-way ANOVA resulted in a significant difference (P<0.05).

Post-hoc test using LSD of two variables (table 3) showed that there was a significant difference of MDA levels for all group comparisons. MDA levels for F70 group was significantly lower compared to that of F100 group (P=0.010), group AL (P=0.000),and group F140. When we compared MDA level for group F100 to group F140 and AL group, the MDA level for group F100 was significantly lower than that of the two other groups (both P=0.000). In other words, AL100 group had a significant lower MDA level than F140 group (P=0.000).

Meanwhile, post-hoc test for 8-oxo-dG level showed that 8-oxo-dG level for group AL was significantly lower compared to group F70 (P=0.000) and group F100 (P=0.000). However, the level of 8-oxo-dG on group AL was not significantly different compared to that of F140 group (P=0.576). The level of 8-oxo-dG was significantly lower for group F140 compared to that of F100 group (P=0.001) and F70 group (P=0.000). 8-oxo-dG level for F100 was significantly lower compared to that of F70

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**Table 2. Experimental data of MDA and 8-oxo-dG**

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>FASTING 70%</th>
<th>FASTING 100%</th>
<th>FASTING 140%</th>
<th>Ad lib 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>weight (gram)</td>
<td>290.80±5.630</td>
<td>292.00±8.456</td>
<td>308.20±4.438</td>
<td>298.00±6.671</td>
</tr>
<tr>
<td>MDA (nmol/mL)</td>
<td>0.770±0.175</td>
<td>1.298±0.889</td>
<td>3.924±0.378</td>
<td>2.418±0.383</td>
</tr>
<tr>
<td>Shapiro-wilk</td>
<td>0.585</td>
<td>0.777</td>
<td>0.998</td>
<td>0.942</td>
</tr>
<tr>
<td>Levene test</td>
<td>0.129</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>8-oxo-dG (ng/mL)</td>
<td>5.040±0.207</td>
<td>3.260±0.207</td>
<td>2.660±0.207</td>
<td>2.580±0.258</td>
</tr>
<tr>
<td>Shapiro-wilk</td>
<td>0.754</td>
<td>0.754</td>
<td>0.754</td>
<td>0.501</td>
</tr>
<tr>
<td>Levene test</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

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**Table 3. Post-Hoc LSD test for MDA and 8-oxo-dG**

<table>
<thead>
<tr>
<th>No.</th>
<th>GROUPS</th>
<th>MDA difference</th>
<th>P</th>
<th>8-oxo-dG difference</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Ad lib vs fasting 140%</td>
<td>-1.506</td>
<td>0.000</td>
<td>-0.080</td>
<td>0.576</td>
</tr>
<tr>
<td>2.</td>
<td>Ad lib vs fasting 100%</td>
<td>1.120</td>
<td>0.000</td>
<td>-0.680</td>
<td>0.000</td>
</tr>
<tr>
<td>3.</td>
<td>Ad lib vs fasting 70%</td>
<td>1.648</td>
<td>0.000</td>
<td>-2.460</td>
<td>0.000</td>
</tr>
<tr>
<td>4.</td>
<td>Fasting 140% vs fasting 100%</td>
<td>2.626</td>
<td>0.000</td>
<td>-0.600</td>
<td>0.001</td>
</tr>
<tr>
<td>5.</td>
<td>Fasting 140% vs fasting 70%</td>
<td>3.154</td>
<td>0.000</td>
<td>-2.380</td>
<td>0.000</td>
</tr>
<tr>
<td>6.</td>
<td>Fasting 100% vs fasting 70%</td>
<td>0.528</td>
<td>0.010</td>
<td>-1.780</td>
<td>0.000</td>
</tr>
</tbody>
</table>
The relationship between MDA and 8-oxodG levels was analyzed using Pearson's correlation test. Pearson's correlation coefficient showed that there was a strong negative relationship \((r=-0.737, P=0.000)\) between two variables (Table 2). This result indicates a reverse action of MDA and 8-oxodG productions, the lower MDA level, the higher 8-oxodG is produced.

**Discussion**

The positive effects of fasting is assumed to correlate with a reduction in oxidative stress related biomarkers attributed to a reduction in the calorie intake. This study investigated the effect of a thirty percent dietary restriction on the oxidative stress, a marker of degenerative diseases, induced in plasma and urine under simulated intermittent/alternate day fasting (ADF) rat model accompanied by caloric restriction. This was done in an attempt to show a beneficial effect of fasting when there is reduction in calorie intake for those partaking ADF. A caloric restriction was applied in order to counteract the increased oxidative stress associated with the increase in caloric intake (sugary food and drinks) during non-fasting (6). This present study showed that thirty percent caloric restriction implemented in these animals significantly decreased MDA levels in plasma of animal subjected to fasting with calorie-restricted at the end of the experiment.

This present study showed that 30% CR with ADF group had the lowest MDA level \((0.770 \text{ nmol/mL})\) compared to other groups. Meanwhile, the highest MDA level was found in 140% calories-fasting group \((3.924 \text{ nmol/mL})\) followed by 100% calories-ad libitum group \((2.418 \text{ nmol/mL})\) and 100% calories-fasting group \((1.298 \text{ nmol/mL})\) respectively. The present findings are consistent with the findings of the previous study investigating the effect of 80% CR diet on SD rats showing that rats subjected to 80% CR diet for 15 days had a lower MDA level compared to those subjected a higher calories consumption per day. The eighty percent CR diet for 2 weeks was able to alter the oxidative stress at cell membranes (13). The alteration of oxidative stress process at cell membrane (lipid peroxidase) is indicated by the level of cell membrane-oxidative stress indicator, MDA (8). This effect could result from The reduction of calorie intake or calorie restriction that have been shown to down regulate metabolic rate causing a reduction of oxygen level and proton leakages at mitochondria (14). In consequence, the production of ROS at complex I and III will be reduced, and thus decreasing oxidative stress process, including at cell membrane that is indicated by a decrease of lipid peroxidase and MDA production (15, 16). In this study, fasting with 30% CR has been shown to reduce MDA level significantly on SD rats.

However, the thirty percent caloric restriction applied on these animals significantly increased urinary 8-oxodG levels of fasting rat with calorie-restricted at the end of the experiment. In contrast to the effect of 30% caloric restriction on MDA finding, the lowest level of urinary 8-oxodG level was found in AL \((ad \text{ libitum})\) group \((2.580 \text{ ng/mL})\), followed by F140 \((2.660 \text{ ng/mL})\), F100 group \((3.260 \text{ ng/mL})\), and F70 group- \((5.040 \text{ ng/mL})\). This finding is different from that of study conducted by Wolfa et al.'s on SD rats aged 4 months with 60% CR. Wolfa et al found that the level of 8-oxodG level rats with 60% CR was decreased in many tissues, including brain, heart, liver, skeletal muscles, intestines, and lymphocytes (17). Another different finding was reported by a study of Stuart and his colleagues on rats aged 8 weeks with 60% CR for 14 months showing a decrease of DNA repair activity at the mitochondria in brain and kidney and, conversely, an increase at the mitochondria in liver. They also found that oxidative and mutation rate were decreased due to CR, but Base Excision Repair (BER) was not affected. Instead, there was an increase of BER nuclear activity in kidney about 26% (18). This is possible that this contradicting effect of urinary parameter on oxidative stress is related to the more effective DNA repair mechanism. In other words, although CR can reduce ROS production, however, at the same time it also increases the efficiency of DNA repair activities, for examples Nuclear Excision Repair (NER), BER, and double-strand break repair, and thus maintaining genomic stability. The concentration of modified nucleoside in urine reflects DNA damages on organism's body, and 8-oxodG at DNA cell reflects a balance between the breakdown and recovery processes (19). It is widely known that CR could increase sirtuin 1.
(SIRT1), a cellular metabolism regulator, that will cause deacetylation at oxoguanine DNA glycosylase (OGG1) enzyme, leading to increase the excision of 8-oxodG site (20). SIRT1 is also known to cause deacetylation to Forkhead transcription factors (FOXO) 3, increasing the expression of several anti-oxidant enzymes, and helping the activities of DNA repair (21).

An excessive calorie intake is assumed to accelerate cell aging. In consequence the repair ability of 8-oxodG will be decreased due to several factors. For examples, aging could cause inability in transporting OGG-1 to cell core compartment as well as to mitochondria. Oxidative stress due to aging could also cause the alteration of OGG1 activities in subcellular targeting. Another mechanism by which aging could decrease 8-oxodG repair ability is because aging causes epigenetic changes through post-translational modification, and decreases the 8-oxodG's incision activities (22). Therefore, this present study suggest that besides increasing 8-oxod-G level, it is assumed that 30% CR might have driven DNA repair activities to be more efficient.

In this study, we found that the fasting rat with 30% CR group had the lowest average weight (9290.80 gr) compared to the other groups. This finding is quite similar with that of the previous study on male Wistar rats aged 3 months with 40% CR showing a reduction in body weight compared to the rats fed in ad libitum diet regimen (23). In a human study, adults subjected to CR with different composition also showed a significant reduction in their body weight (24). Thus, 30% CR has been shown to reduce body weight significantly. However, to gain insight into the mechanism of 30% CR effects on oxidative stress indicators, the activity of SIRT1, OGG1, FOXO3, and BER.

**Conclusion**

The Intermittent fasting (IF) accompanied with 30% CR decreases MDA level, but not 8-oxodG level. This finding suggest the need for calorie restriction for fasting subjects. In other words, the beneficial effect of fasting requires decrease in overall caloric intake. The health benefit effect of fasting as a form of dietary restriction are not simply the result of the restriction of feeding time, but can be achieved by reduction in caloric intake. Further studies are important to investigate the effect of CR on SIRT1, OGG1, FOXO3, and BER to gain insight into the mechanism of caloric restriction.

**Acknowledgments**

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**References**


