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A pilot study into the effects of various dietary restriction schedules in rabbits.

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Introduction.

Ad libitum feeding of rabbits leads to obesity, which can be considered undesirable from a scientific as well as from an animal welfare point of view. Restricted feeding is known to promote long-term health in rodents, as it increases survival time, prevents obesity, reduces and/or postpones the occurrence of cancer and prevents chronic kidney disease as compared to ad libitum feeding *(Keenan & Soper 1995; Turnbull et al. 1985)*. When applying a dietary restriction schedule, the amount of food can be restricted in amount and/or time. The question arises which feeding schedule would be best from a scientific as well as an animal welfare point of view. Feeding a restricted amount of diet in the afternoon instead of in the morning, significantly reduced abnormal behaviour being recorded during the observation period in the dark hours *(Krohn et al. 1999)*. Feeding restrictedly in the afternoon also led to a significantly lower occurrence of abnormal behaviour as compared to ad libitum feeding *(Krohn et al. 1999)*. These behavioural studies indicate that feeding a restricted amount of food at the "proper" time of day, may improve rabbit welfare in the laboratory. The effects of various dietary restriction regimes on physiological and biochemical parameters in the rabbit were evaluated in our study.

Materials and Methods.

Animals.

8 female and 4 male French long-eared outbred rabbits of about 3 months of age were used. The rabbits had originally been bought from a commercial meat production farm (no health certificate available) and further bred as an outbred colony at Odense University. The animals were clinically healthy and remained so during the experiment. Each rabbit had an individual number as ear tattoo. Animals were housed individually in metal cages with grid floors. Environmental conditions: temperature 20±3^{0}C; relative humidity 50±10%; lights on from 6.00 - 18.00 hrs. Rabbits were acclimatised during a 3 week period in which they were fed a chow diet ad libitum (Altromin 2020, Chr. Petersen A/S, Ringsted, Denmark). Daily food- and water intake were registered. The animals were divided into 4 groups on the basis of body weight and sex (2 females and 1 male per group) at the end of the acclimatisation period (day 0). The groups were divided at random over the rack. The experimental scheme was as follows:

Group "AL":
Ad Libitum feeding - rabbits could eat as much as they liked, 24 hrs per day, fresh food was supplied each morning (7.30 hr).

Group "FR":
Food Restriction - 60% of the amount eaten by group "AL" was fed each morning (7.30 hr).

Group "NFR":
Night Food Restriction - rabbits got ad libitum food from 7.30-14.30 hr.

Group "DFR":
Day Food Restriction - rabbits could eat ad libitum from 14.30-7.30 hr.

Animals were not yet divided in experimental groups during the acclimatisation period, however,

acclimatisation results were afterwards calculated per experimental group by using the individual ear numbers. These results were included in the results tables.

The experimental period lasted 6 weeks. Body weight was measured each week or every 2nd. week and food and water intake were measured every day. Blood was collected at days -7, 7 14, and 35 (between 7.30-8.30 hr). Animals were sedated with 0.2-0.3 ml Hypnorm intramuscularly before blood collection of 8 ml per animal. Animals were fasted from 14.30 hrs. the day before, for the blood collections on days -7 and 14, however the animals from the group DFR were allowed to eat from 13.00-14.30, in order to avoid a 24 hr. fasting period. Animals were not fasted for the collections on days 7 and 35. On day 42 the animals were killed by a hit in the occipital region with subsequent exsanguination. Then autopsy was performed. The sequence of collection of blood samples and killing was randomised throughout the groups.

Blood parameters.

Blood was collected in EDTA-containing tubes and centrifuged. The plasma was frozen at -20°C until further analysis for total cholesterol, creatinin, triglycerides, urea, GGT, ALT, AST and ALP. Total cholesterol and ALP were analyzed in duplo on a spectrofotometer (Shimadzu UV-160A), always within 10 days after the samples had been frozen. All other samples were analysed simultaneously in duplo on an automatic auto-analyzer (Cobas Mira, Roche, Switzerland) for the remaining parameters; the maximum storage period until analysis was 50 days. One or two commercially available control sera (when available) were analyzed per parameter in the same run as the animal samples. In case the results of these controls were outside the given range provided by the manufacturer, the whole run was repeated. Results were accepted when controls were within the given range.

Total cholesterol was measured at 37°C and 500 nm wavelength, by using a test-kit from Boehringer Mannheim (#MPR 2 1442 350). Seronorm (Nycomed Pharma, Roskilde, Denmark) and Calibrator K-92E.10 (Nycomed) were used as control sera. ALP was measured at room temperature and 405 nm wavelength by using a Boehringer Mannheim testkit (MPR 3 782 874/782 858). It cannot be excluded that the use of EDTA may have inhibited ALP enzyme activity, as mentioned in the manufacturer's information. Seronorm and Calibrator K-92E.10 were used as controls. For ALT, AST, GGT, urea, triglycerides and creatinin, control sera (Ultimat 3, control N and P serum) as well as some of the test kits (Unimate 5 ALT, Unimate 5 AST, Unimate 5 Urea, Unimate 5 Trig) were obtained from Roche Diagnostics (Hvidovre, Denmark). The remaining test kits were obtained from Boehringer Mannheim (gamma-GT, Creatinine PAP).

Section parameters.

When autopsied, a full macroscopic inspection was carried out on each animal. No macroscopically visible lesions were found. The liver was removed from the animal and weighed. Both kidneys were weighed and the total kidney weight per animal was used. The intestinal tract was ligated around the oesophagus, just before the entrance into the stomach and directly before the anus and taken out and weighed. The stomach and intestines were then opened by cutting them open longitudinally and the contents removed by rinsing them in running tap water. Then the intestinal tract was weighed again and the intestinal contents calculated.

Statistics.

Groups were compared by using the one-way analysis of variance (ANOVA: dietary regime was the variable tested). In case the ANOVA test indicated a significant difference ($P<0.05$), individual groups were compared by using Tukey B test. A significant difference between individual groups was considered to be present, when $P<0.05$.

Results

Animal parameters

Body weights in the FR and NFR groups were significantly lower than those in the DFR group after 1, 2 and 6 weeks (Table 1). After 5 weeks

Table 1. Body weight (BW), growth, food-, water intake and food conversion

Groups[1] Parameter	AL	FR	NFR	DFR	ANOVA (P)
BW t = -2 wk (g)	2033±145	2082±142	2149±130	2270±173	
BW t = -1 wk (g)	2721± 73	2827±118	2837± 71	2954±121	
BW t = 0 (g)	2862± 74	3009±170	2939± 48	3108±119	
BW t = 1 wk (g)	3256± 92^{ab}	3055±183^{a}	2994± 64^{a}	3490±107^{b}	<0.05
BW t = 2 wk (g)	3428±150^{ab}	3184±173^{a}	3158±71^{a}	3718±119^{b}	<0.05
BW t = 5 wk (g)	3936±140^{a}	3468±149^{b}	3577± 46^{b}	4223± 56^{c}	<0.05
BW t = 6 wk (g)	4093±122^{a}	3563±178^{b}	3685± 14^{b}	4344± 51^{a}	<0.05
Water intake t = 1-6 wk (g/d)	255± 34^{a}	381± 46^{b}	244± 44^{a}	284± 6^{a}	<0.05
Food intake t = 1-6 wk (g/d)	206± 18^{a}	125± 0^{b}	126± 3^{b}	213± 7^{a}	<0.05
Growth t = 0-6 wk (g/d)	30.1±1.6^{a}	13.5±0.3^{b}	18.2± 1.5^{b}	30.1± 3.0^{a}	<0.05
Food conversion (g food per day/ g growth per day)	6.8±0.3^{a}	9.3±0.2^{b}	6.9±0.5^{a}	7.1± 0.7^{a}	<0.05

Means and SD are given. 3 rabbits per group.
[1] The explanation of AL, FR, NFR and DFR is given in the Materials & Method section.
^{abc}Results in the same row, not bearing the same superscript, are significantly different.

were the body weights in the FR and NFR groups significantly lower than those in the DFR and AL group. Body weight in the DFR group was significantly higher than that in the AL group after 5 weeks only. The rabbits of the FR group drank significantly more water as compared to those in the other 3 groups (Table 1).

Food intakes in the FR and NFR groups were significantly lower than those in the AL and DFR groups (Table 1). Similar food intakes were measured in the AL and DFR groups.

A significantly lower growth rate occurred in the animals of groups FR and NFR as compared to those in the AL and DFR groups (Table 1). As expected on the basis of food intake measurements, had AL and DFR fed rabbits similar growth rates. Food conversion in the FR group was significantly higher than those in the other 3 groups (Table 1).

Blood parameters.

Creatinin concentrations were significantly higher in the FR and NFR groups than those in the AL and DFR groups after 7 and 35 days (Table 2). After 14 days was the creatinin concentration significantly higher in the FR group only as compared to the AL and DFR groups (Table 2).

Urea concentrations in the FR and NFR groups were significantly lower than those in the AL and DFR groups after 14 days (Table 2). No significant differences were measured at the other time points. No significant group differences were found for total cholesterol, triglycerides, ALT, AST and GGT concentrations (Table 2). ALP was significantly higher in the FR group as opposed to the other 3 groups before the start of the experimental period (Table 2, day -7). A significantly lower ALP concentration was found in the AL group as compared to the other 3 groups after 7 days (Table 2).

Section parameters

Absolute and relative liver weight and relative kidney weight did not differ between the groups (Table 3). Absolute kidney weights of FR and NFR rabbits were significantly lower than those of the DFR animals (Table 3). A significantly lower absolute kidney weight was seen in the FR group as compared to the AL group (Table 3). Intestinal weights without contents were similar in all groups (Table 3). Absolute and relative weights of the contents from the intestines were significantly higher in the FR and NFR groups as compared to those of the AL and DFR groups (Table 3).

Discussion

Similar food intakes of rabbits in the DFR and AL groups were measured. Thus, when DFR rabbits were offered food that was freely available from 14.30 - 7.30 hr each day, a similar amount was ingested as rabbits that were under ad libitum feeding conditions for 24 hours per day. These results indicate that although the DFR feeding schedule provides a food restriction over time, it does not lead to a reduced food intake or lowered body weight. This might have been expected on the basis that food was provided during the normal eating period of rabbits (early evening and night, *Hörnicke et al. 1984*) and during a period as long as 17 hours per day. As determined by the experimental design was the food intake in the FR group significantly lower than that in the AL group, which resulted in a lowered body weight. Providing food freely during 7.30-14.30 hr (NFR group) led to similar food intakes and body weights as in the FR group. A lower food intake in the NFR group as compared to that in the DFR group can be explained in two ways. Firstly, the usual eating time of rabbits is around evening and night, and, secondly, the food was available during a shorter period (NFR group 7 hours during the day; DFR group 17 hours during the evening and night). Although rabbits obtained a lower body weight by restricting the amount of food (FR) and hours (NFR), the eating periods were outside the natural feeding time of the rabbit. There are indications that feeding a restricted amount of food in the afternoon instead of in the morning, may improve rabbit's welfare *(Krohn et al. 1999)*.

Food conversion in the FR group was significantly higher than in the other 3 groups. The explanation for this is not clear, particularly when one compares the results to those of the NFR group, which had a similar food intake level during the daytime. Our subjective impression was that the

Table 2. Blood parameters

Groups[1] Parameter	AL	FR	NFR	DFR	ANOVA (P)
Creatinine					
t = -7d (µmol/L)	75.3±6.5	86.3±1.5	82.7±9.0	79.3±6.8	
t = 7d (µmol/L)	77.7±7.1[a]	105.5±11.5[b]	103.3±7.5[b]	79.7±4.7[a]	<0.05
t = 14d (µmol/L)	87.0±7.8[a]	113.7±5.8[b]	99.3±9.1[ab]	92.7±4.0[a]	<0.05
t = 35d (µmol/L)	87.7±4.7[a]	117.7±6.4[b]	110.3±10.4[b]	89.4±1.4[a]	<0.05
Urea					
t = -7d (mmol/L)	7.8±0.5	7.8±2.1	9.3±1.2	7.5±0.9	
t = 7d (mmol/L)	5.4±0.3	5.0±0.6	5.5±1.0	5.8±0.7	
t = 14d (mmol/L)	8.5±0.9[a]	5.2±0.1[b]	5.4±0.3[b]	8.1±0.6[a]	<0.05
t = 35d (mmol/L)	6.2±0.9	5.9±0.7	5.7±0.5	6.9±0.4	
Triglycerides					
t = -7d (mmol/L)	1.5±0.3	0.8±0.2	1.4±0.4	1.3±0.7	
t = 7d (mmol/L)	1.1±0.2	0.7±0.1	0.8±0.3	0.9±0.6	
t = 14d (mmol/L)	1.0±0.2	0.7±0.1	0.9±0.3	1.0±0.3	
t = 35d (mmol/L)	0.9±0.3	0.5±0.1	0.8±0.3	1.0±0.2	
Total cholesterol					
t = -7d (mmol/L)	1.4±0.2	1.5±0.1	1.8±0.2	1.5±0.2	
t = 7d (mmol/L)	1.8±0.3	1.5±0.4	2.2±0.8	1.7±0.3	
t = 14d (mmol/L)	1.8±0.3	1.4±0.3	2.0±0.7	1.8±0.2	
t = 35d (mmol/L)	1.5±0.3	1.4±0.3	1.5±0.4	1.7±0.3	
ALP					
t= - 7d (U/L)	57,3±12.1[a]	100.3±18.4[b]	42.3±2.9[a]	30.0±7.8[a]	<0.05
t = 7d (U/L)	26.0±1.0[a]	56.0±7.5[b]	55.3±11.1[b]	55.7±13.6[b]	<0.05
t = 14d (U/L)	19.7±1.2	30.7±7.4	24.7±1.5	21.3±2.1	
t = 35d (U/L)	17.4±2.4	29.7±6.8	39.9±23.0	21.7±7.9	

Table 2 continued

Groups[1] Parameter	AL	FR	NFR	DFR	ANOVA (P)
ALT					
t = -7d (U/L)	37±29	25±17	31±10	37±10	
t = 7d (U/L)	25±8	17±2	22±1	24±3	
t = 14d (U/L)	29±13	22±5	21±0	31±6	
t = 35d (U/L)	30±15	23±2	28±4	31±6	
AST					
t = - 7d (U/L)	25±14	27±6	24±6	28±7	
t = 7d (U/L)	30±10	22±4	26±2	23±10	
t = 14d (U/L)	30±7	28±9	28±6	28±11	
t = 35d (U/L)	28±10	37±12	26±2	26±5	
GGT					
t= - 7d (U/L)	8±1	8±1	9±2	6±1	
t = 7d (U/L)	7±2	8±1	8±1	6±2	
t = 14d (U/L)	8±1	10±2	8±2	7±2	
t = 35d (U/L)	6±0	6±3	7±3	6±1	

Means and SD are given. 3 animals per group.

Fasting blood was collected on days 1 and 21.

Non-fasting blood was collected on days 14 and 42.

[1] The explanation of AL, FR, NFR and DFR is given in the Materials & Methods section.

[ab] Results in the same row, not bearing the same superscript, are significantly different.

Table 3. Autopsy data

Groups[1] Parameter	AL	FR	NFR	DFR	ANOVA (P)
Liver wt (g)	121.6±6.8	107.0±12.8	121.9±16.4	129.3±6.2	
Liver wt (% of BW)	3.0±0.2	2.9±0.3	3.3±0.4	3.0±0.1	
Kidney wt (g)	22.1±1.0 ^{ab}	18.4±0.6^{c}	19.8±0.8^{bc}	24.1±1.1^{a}	<0.05
Kidney wt (% of BW)	0.5±0.03	0.5±0.02	0.5±0.02	0.6±0.03	
Wt intestines without contents (g)	244±28	228±32	223±6	257±36	
Wt intestines without contents (% of BW)	5.9±0.5	6.2±0.7	6.0±0.1	5.9±0.8	
Intestinal contents (g)	207±39^{a}	376±43^{b}	387±49^{b}	195±3^{a}	<0.05
Intestinal contents (% of BW)	5.0±0.9^{a}	10.3±1.3^{b}	10.4±1.3^{b}	4.5±0.1^{a}	<0.05

Means and SD are given. 3 animals per group.
[1] The explanation of AL, FR NFR and DFR is given in the Materials & Methods section.
^{abc} Results in the same row, not bearing the same superscript, are significantly different.

FR rabbits, that received a restricted amount of food in the morning, had eaten all their food before noon, whereas the animals in the NFR group divided food ingestion over the entire period (7.30-14.30). When taking away the diets at 14.30 hrs, the NFR rabbits appeared quiet, whereas the FR rabbits gave a nervous impression, walking back and forth in the cage. It might be that the FR rabbits quickly consumed the restricted amount of food given (fear for competitors?), leaving them seeking for more food during the rest of the time of day and night. Providing a larger amount of food in the NFR rabbits appeared not to lead to fast consumption (no fear of competition, because the amount sufficed for more than one animal?). A higher activity pattern is expected to lead to a higher food conversion, as more energy of the food is used for physical activity. A higher activity might also explain the significantly higher water intake in the FR group. Duffy et al. (1989) discussed that water consumption in restricted rats was probably important to help in partially filling the stomachs, thereby relieving hunger and compensating for the lack of food. A high water intake in the FR group might relate to the expected "shortage" of food during a large time of the light and dark hours.

Different feeding regimens in this study did not influence blood lipid parameters (triglycerides and total cholesterol), nor did they influence liver enzymes (ALT, AST, GGT). The reason for the significantly higher ALP-value in the FR group on day -7 is not clear. The high ALP level in the FR group had disappeared on day 7. An explanation for the significantly lower ALP concentration in the AL group on day 7 cannot be given, other then a relatively quicker lowering with age based on the fact that these AL rabbits had been accustomed to the dietary schedule 3 weeks longer than the other groups.

The significantly higher creatinin concentrations in the FR and NFR groups may be the result of a (temporarily?) decreased kidney function and/or food searching activity during the night, leading to more release of creatine from the muscles which is measured in the creatinine assay as well.

Collecting fasted (t= -7d and 14 d) or non-fasted blood did not appear to influence the levels of blood parameters, with the possible exception of the urea concentrations at day 14 (Table 2). Significantly higher urea values were found in the AL and DFR groups after 14 days only, when fasted blood samples were collected. The collection of fasted instead of non-fasted blood samples, might have been the cause for different urea concentrations in the various groups. Higher blood urea concentrations can indicate a diminished kidney function but can also be induced by diet (protein) ingestion. It was likely that higher blood urea concentrations after fasting was related to altered kidney function, as the animals had not eaten for a period of 18 hrs. It is well-known from rodent studies that ad libitum feeding leads to an early occurrence of nephropathies *(Keenan & Soper 1995)*.

Absolute kidney weights in the FR and NFR groups were lower than in the AL and DFR groups, but this was related to higher body weights, as there were no differences in relative kidney weights. The non-fasted animals were killed during daytime (groups in random order), which led to significantly higher weights of intestinal contents in the FR and NFR groups, as these animals had received new food in the morning after overnight fasting. DFR and AL rabbits were expected to eat during the early evening and night, because DFR rabbits received food only during that period and AL animals were expected to eat during the "natural" eating hours. A reduced intestinal contents in the morning would be the result. The weights of the intestines without contents was similar in all groups, indicating that no obvious hypertrophy had occurred.

In conclusion: The provision of a restricted amount of food equalling to 60% of the ad libitum intake in the morning, led to a reduction in body weight as compared to ad libitum intake. However, providing this restricted amount also caused a higher water intake and higher food conversion. Having food freely available during a 7-hr period during daytime, led to a restricted food intake (60% af ad libitum intake), without increasing water intake or food conversion. Giving an unlimited amount of food during a 17-hr period per day, which included the dark period, led to the same food intake and body weight as ad libitum feeding for 24 hours per day. The withdrawal of food during the dark hours (FR and NFR groups), led to significantly higher blood creatinin concentrations. Whether this could be related to a higher physical activity induced by food searching activity during the dark hours, needs further study. The possibly positive effects of food restriction by amount or time (e.g. 7 hours) during the dark hours will be evaluated in future studies.

Summary

Ad libitum feeding in rabbits quickly leads to obesity. In order to prevent obesity, various dietary restriction schedules were studied for their effects on body weight, food and water intake, food conversion, blood lipid parameters (triglycerides, total cholesterol), kidney function (blood urea and creatinine), enzymes (ALP, ALT, AST, GGT) and various autopsy parameters. In this pilot study the effects of ad libitum feeding (AL) were compared to feeding 60% of the ad libitum amount in the morning (FR), having food freely available during working hours (7.30-14.30 hr; NFR) and in between working hours (14.30-7.30 hr; DFR). The DFR schedule did not give reduced food intakes nor body weights as compared to the ad libitum feeding, and can therefore not be regarded as food restriction. The food intake in the NFR group was comparable to the food intake in the FR group and both obtained food during daytime. However, there was a significantly higher food conversion and water intake in the FR group only. The reason for this is unclear. Blood creatinine values were significantly higher in the FR and NFR groups. Whether this was related to food searching activity during the dark hours, leading to creatine release from muscle tissue, needs further study.

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