Applied Physiology, Nutrition, and Metabolism
 Physiologie appliquée, nutrition et métabolisme

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Journal:	<i>Applied Physiology, Nutrition, and Metabolism</i>
Manuscript ID	apnm-2016-0301.R1
Manuscript Type:	Article
Date Submitted by the Author:	16-Sep-2016
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Keyword:	High protein diet, obesity, renal damage, renal hypertrophy, kidney health

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Mixed compared to single source proteins in high protein diets affect kidney structure and function differentially in obese *fa/fa* Zucker rats

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Abstract

Questions remain regarding the potential negative effects of dietary high protein (HP) on kidney health, particularly in the context of obesity in which the risk for renal disease is already increased. To examine whether some of the variability in HP effects on kidney health may be due to source of protein, obese *fa/fa* Zucker rats were given HP (35% of energy from protein) diets containing either casein, soy protein or a mixed source of animal and plant proteins for 12 weeks. Control lean and obese rats were given diets containing casein at normal protein (NP, 15% of energy from protein) levels. Body weight and blood pressure were measured, and markers of renal structural changes, damage and function were assessed.

Obesity alone resulted in mild renal changes, as evidenced by higher kidney weights, proteinuria and glomerular volumes. In obese rats, increasing the protein level using the single, but not mixed, protein sources resulted in higher renal fibrosis compared to the lean rats. The mixed protein HP group also had lower levels of serum monocyte chemoattractant protein-1, even though this diet further increased kidney and glomerular size. Soy and mixed protein HP diets also resulted in a small number of damaged glomeruli, while soy compared to mixed protein HP diet delayed the increase in blood pressure over time. Since obesity itself confers added risk of renal disease, an HP diet from mixed protein sources that enables weight loss but has fewer risks to renal health may be advantageous.

Keywords:

High protein diet, protein source, obesity, renal damage, renal hypertrophy, kidney health, fibrosis

1. Introduction

The worldwide upsurge in the prevalence of obesity (Ng et al. 2014) has resulted in increased interest in high protein (HP) diets as a strategy for weight loss and management of metabolic syndrome components and cardiovascular disease (Bantle et al. 2008, Lau et al. 2007, Lacroix et al. 2004, Clifton et al. 2009). A diet providing over 25% of energy as protein is generally considered HP, whereas a normal protein (NP) diet supplies about 15% of energy from protein (Clifton and Keogh. 2007). HP diets may mediate their beneficial effects by suppressing hunger and increasing energy expenditure (Eisenstein et al. 2002), thereby reducing body weight and fat mass while retaining lean mass (Farnsworth et al. 2003, Claessens et al. 2009). However, there is evidence for potential detrimental effects of HP diets on renal health [reviewed in (Marckmann et al. 2015) and (Friedman. 2004)]. Increased protein intake is associated with renal enlargement (Hammond and Janes. 1998, Fernandez-Repollet et al. 1989) and increased glomerular filtration rate (GFR) in humans (Juraschek et al. 2013, Skov et al. 1999) and in animals (Hostetter et al. 1986). Adverse effects of HP on the kidney is a particularly important concern in obese populations due to their already compromised kidney health (Henegar et al. 2001, Wickman and Kramer. 2013). When overweight and obese human subjects consume an HP diet for 6 months their kidneys are enlarged and GFR is elevated (Skov et al. 1999). Whether these structural adaptations are detrimental to the kidney in the long-term, however, is not clear as there are indications from partially nephrectomized patients that renal enlargement and elevated GFR might be beneficial adaptive changes (Sugaya et al. 2000, Higashihara et al. 1990, Regazzoni et al. 1998). Renal enlargement and increased GFR in animals given HP diets long-term is accompanied by histological damage and increased proteinuria (Jia et al. 2010, Aparicio et al. 2013, Wakefield et al. 2011, Hostetter et al. 1986). However, it is also important to note that

there are a number of studies in normal and obese subjects where no detrimental effects of HP on kidneys were observed (Lacroix et al. 2004, Knight et al. 2003, Claessens et al. 2009, Friedman et al. 2012).

One of the reasons why varied results are observed with HP diets may be due to differences in the type of protein employed. Some studies have used animal proteins, others have used plant proteins, while others have used a mixture of several proteins. Plant based proteins have different effects on kidney compared to animal based proteins, as demonstrated by an increase in urinary albumin secretion with casein but not with soy protein at HP levels in rats (Wesson et al. 2007). In healthy young males, animal based protein consumption results in higher urinary protein secretion and GFR compared to plant based protein intake at equal levels (Kontessis et al. 1990). Further, a large cross sectional study found a positive association between albuminuria and protein intake for animal but not plant protein consumption (Toeller et al. 1997). Another recent observational study found that blood urea nitrogen concentration increased with higher animal and dairy protein consumption, but not with plant proteins. However, this study also reported that GFR and blood creatinine levels were not associated with any protein source (Berryman et al. 2016). A large Chinese prospective cohort study found that while red meat consumption strongly associated with ESRD risk in a dose- dependent manner, consumption of poultry, fish, eggs, or dairy products did not associate with risk of ESRD (Lew et al. 2016). Interestingly, another human study found no difference in enhancement of GFR between an acute load of beefsteak or baked soy (Orita et al. 2004). Effects of protein intake on renal hemodynamics appear to be highest with animal proteins, followed by milk proteins and then plant proteins (Kontessis et al. 1990, Wiseman et al. 1987, Bilo et al. 1989). However, dietary protein intake of humans comes from a mixed source of animal and plant proteins, and to our knowledge there has

been no study investigating the renal effect of type of protein in HP diets in the context of obesity. Therefore, the objective of this study was to investigate the effect of dietary HP and to compare the effects of mixed versus single sources of protein in HP diets on kidney health in obese rats.

We have previously reported that an HP diet composed of mixed proteins (compared to single protein sources of casein or soy protein) was more effective in reducing insulin resistance and hepatic steatosis independent of weight loss in the obese *fa/fa* Zucker rat (Wojcik et al. 2016). That report demonstrated that the source of protein within an HP diet is critical for the management of these metabolic syndrome parameters. In the current study, we report on the effect of these protein sources on renal health in these same rats provided HP diets.

2. Materials and Methods:

2.1 Animals and Diets

All animal procedures were approved by the University of Manitoba Animal Care Committee and were in accordance with the guidelines of the Canadian Council on Animal Care. To test the effect of protein source in an HP diet on kidney structure and function, 13 week old male obese *fa/fa* Zucker rats (Charles River, St-Constant, QC, Canada) were randomly assigned to either one of three HP (35% of energy from protein) diets [containing casein (faHC), soy protein (faHS) or a mixed source (faHM, consisting of equal amounts of protein from egg white, milk protein, gluten and soy protein)] or were provided an NP (15% of energy from protein) diet with casein (faNC) as the source of protein, for 12 weeks. The obese *fa/fa* Zucker rat is a genetic

model of obesity that harbors a mutation in the leptin receptor gene (Phillips et al. 1996), causing dysregulated food intake control and obesity (Chua et al. 1996). Although glomerular hypertrophy and other renal complications develop after 10 weeks of age in these rats (Coimbra et al. 2000), a previous study in diet-induced obese rats showed that effects of HP diets occur after 8 weeks of feeding (Devassy et al. 2015). Control lean Zucker rats (InNC) also were provided the NP diet. The mixed source of protein was used to mimic animal/plant protein consumption ratios by humans (Smit et al. 1999). The diets were based on the AIN-93M diet (Reeves et al. 1993), with the HP diet differing from the NP diet by exchanging equal calories from carbohydrate for protein (Table 1). Twelve rats were randomly assigned to each diet group, were housed singly in cages in a temperature- and humidity-controlled environment with a 12 hour day/night cycle and were given free access to water and diet.

2.2 Animal procedures

Food intake and body weights were measured weekly. At the beginning of the 12th week of feeding, rats were placed in metabolic cages for 3 days and the urine was collected on the third day. Blood pressure was measured in conscious rats at week 3, 7 and 11 using a multichannel blood pressure system with a tail-cuff sphygmomanometer (Coda 6 System, Kent Scientific, Torrington, Conn), as described previously (Cipolla et al. 2006). After 12 weeks, the rats were fasted overnight (12 h), anaesthetized with isoflurane and exsanguinated via cardiac puncture to obtain blood samples for serum. Kidneys were removed, weighed and the left kidney was sectioned in half longitudinally across the hilum, with one half fixed in 10% buffered formalin for histological analyses.

2.3 Biochemical analysis

Serum and urinary creatinine were measured using the Jaffe reaction as modified by Heinegard & Tiderstrom (Heinegard and Tiderstrom. 1973), and adapted for micro assay. Urine protein was determined using the Bradford protein assay method with bovine serum albumin as a standard (Bradford. 1976). An enzyme-linked immunosorbant assay was used to determine serum monocyte chemoattractant protein-1 (MCP-1) (Life Technologies Inc., ON, Canada). MCP-1 is a chemokine produced by the tubular epithelial cells (Prodjosudjadi et al. 1995); its level is a useful tool to assess renal injury (Tesch. 2008, Morii et al. 2003).

2.4 Histology and image analysis

Left kidneys were fixed in formalin and embedded in paraffin, sectioned at 5 μ m and processed using our published methods (Sankaran et al. 2007). For quantification of tubulointerstitial fibrosis, sections were stained with Sirius red (an adaptation of Masson's trichrome stain), which permits image analysis measurement using a standard incandescent microscope light source. 10X magnification images from a standard microscope were captured with a Spot Junior charge-coupled device camera by random stage movement throughout the renal cortex and were analyzed with Image Pro version 6.0 (Media Cybernetics, Silver Spring, MD, USA). An average of twenty-six images per kidney were collected for all histomorphometric analyses, and all measurements were carried out in a blinded fashion. Tubulointerstitial fibrosis was measured by densitometry as described (Sankaran et al. 2004). Transverse tissue sections were stained with hematoxylin and eosin for glomerular diameter measurement and mean glomerular volume was determined with standard stereological techniques developed by Weibel, as described previously

(Sankaran et al. 2007, Caligiuri et al. 2014). Structural damage in the glomeruli was analyzed using an objective scoring system, as previously described (Caligiuri et al. 2014).

2.5 Statistical analysis

Data were analyzed using SAS (SAS Institute, version 9.2, Cary, NC). Normality of the data was assessed using the Shapiro–Wilk statistic and homogeneity of variance was tested using Levene's Test for Homogeneity of Variance. Data that were not normally distributed were log transformed. One-way ANOVA was used to analyze differences between groups (n=10-12 rats/group) for all parameters with one endpoint followed by Duncan's multiple range post hoc test. Repeated measures ANOVA was employed for body weight measurements. Significance limit was set at $P < 0.05$.

3. Results

Obese Zucker rats (faNC) provided the NP diet had higher body weights at all time-points compared to lnNC rats on this diet (Figure 1). Increasing the level of casein did not significantly increase the weight of obese rats (faNC vs faHC), but faHS and faHM rats provided with higher levels of soy and mixed protein, respectively, had higher body weights than the faNC rats from weeks 5 through 12. However, body weights among rats on HP diets were not different. Average daily feed intake was higher in faNC compared to lnNC, but not different among faNC, faHC, faHS and faHM (Table 2).

Kidney weight, glomerular volumes and proteinuria were higher in faNC compared to lnNC rats, indicating that renal alterations and increased risk of renal disease due to obesity were present.

However, kidney weight adjusted for body weight, fibrosis, glomeruli damage, serum MCP-1 and serum creatinine did not differ between these groups, indicating that the damage to the kidneys was relatively minor in these obese rats at this age (Figure 2, Table 2).

Increasing the level of dietary casein in obese rats did not result in more renal damage, as there were no differences in any renal parameters between faHC and faNC rats, although renal fibrosis (pixels/field and %) in the faHC (but not faNC) rats was higher than in the lnNC rats. Higher levels of protein from soy protein had similar effects as casein, but in addition this group also had more damaged glomeruli when compared to the lnNC rats. Rats provided HP diets using a mixed protein source had differences in all renal parameters measured compared to the other groups. In comparison to the lnNC rats, faHM rats had higher kidneys weights, glomerular volumes and proteinuria, similar to the other obese rat groups given HP diets. In addition, faHM kidney weights were higher than in the faNC groups and, their kidney weight adjusted to body weight, number of damaged glomeruli and serum creatinine also were higher compared to the lnNC group. In addition to faHM having higher glomerular volumes compared to lnNC and faNC rats, they also had greater glomerular volumes than faHC and faHS rats. Interestingly, despite effects on these structural parameters in faHM rats, most markers of renal damage were not worse in the faHM rats. In contrast to the faHC and faHS groups, renal fibrosis (pixels/field and %) in faHM rats was similar to the levels in the lnNC group, and was lower than in faHS rats. In addition, serum MCP-1 was lower in faHM rats than in any other lean or obese group of rats.

There were no differences in mean arterial pressure (MAP) between lean and obese rats (lnNC vs faNC), or between obese rats provided casein NP and HP diets (faNC vs faHC) at any of the 3 time points that blood pressure was measured, weeks 3, 7 or 11 (Figure 3). Among obese rats, at 3 and 7 weeks, faHS rats had lower blood pressure compared to faHM rats. At 7 weeks the mean arterial pressure in faHS rats was also lower than in the lnNC rats. By the end of the study, blood pressure in faHS rats had risen to levels comparable to the other groups.

4. Discussion

This study demonstrated that the effect of an HP diet on renal health in obesity is dependent on both the type of protein and on the markers of renal injury being assessed. The differential effects of protein source in HP diets in this study were observed in a relatively early stage of renal disease, with only kidney weight, proteinuria and glomerular hypertrophy being increased in obese rats.

HP diets are often associated with structural alterations in the kidney. Rats and pigs provided HP compared to NP diet long term display enlarged kidneys and glomeruli (Hostetter et al. 1986, Jia et al. 2010, Aparicio et al. 2013, Wakefield et al. 2011). In overweight and obese humans, 6 months of dietary HP also results in kidney enlargement (Skov et al. 1999). In the present study, kidneys and glomeruli were larger in obese rats but were not further enlarged by higher levels of dietary casein or soy protein. However, obese rats given HP from mixed sources had enlarged kidneys compared to obese rats given an NP diet, and larger glomeruli when compared to all

other groups, indicating that the source of protein in an HP diet influences these structural parameters.

Whether these structural changes would eventually result in impaired renal function, however, remains to be determined. Renal hypertrophy and concomitant elevated GFR is observed subsequent to unilateral nephrectomy, suggesting that this process is an adaptive positive response (Sugaya et al. 2000). Additionally, remnant kidney function remains unaffected after more than 20 years of prolonged hyperfiltration in nephrectomized patients (Higashihara et al. 1990, Regazzoni et al. 1998). In healthy, overweight and obese human subjects, albuminuria, a marker of impaired renal function, did not change despite increased renal size and GFR after 6 months of an HP diet (Skov et al. 1999). In our study, the differences in proteinuria or serum creatinine were only present when compared to the lean group, with no apparent effects of protein level or source in this very early stage of obesity-associated renal disease. However, *fa/fa* rats given HP diets containing casein or soy protein had elevated fibrosis compared to lean control rats, while obese rats given a mixed HP diet were no different in this regard compared to the lean or obese rats given the NP diet. Renal interstitial fibrosis is an indicator of damage that is associated with HP diets (Jia et al. 2010) and greater fibrosis was observed in the HP soy protein group. A similar trend for increased interstitial connective tissue deposition was reported previously in Wistar rats given soy protein at HP levels, compared to those given NP diet (Aparicio et al. 2013).

The lower fibrosis in the HP mixed protein group may be related to reduced recruitment of macrophages. Although serum MCP-1 was not higher in obese compared to lean rats in our

study, as may have been expected (Graf et al. 2014), it was lower in the HP mixed protein group compared to all others. MCP-1 is a pro-inflammatory chemokine that recruits macrophages to the kidney (El Nahas. 2006). A reduction in renal MCP-1 levels in normal weight rats given a mixed source of protein at HP levels has been reported previously (Wakefield et al. 2011). This could be beneficial since renal interstitial fibrosis in obesity is mediated in part by MCP-1 (Chow et al. 2007, Tesch. 2008, Morii et al. 2003).

Interestingly, both the HP soy and mixed protein groups had greater numbers of damaged glomeruli, suggesting that both HP diets may have a detrimental impact on this parameter. This is consistent with a study demonstrating a greater prevalence of sclerotic glomeruli when normal weight rats were given a mixed protein HP diet for up to 8 months (Hostetter et al. 1986), but it contrasts with a study in which rats provided dietary HP soy protein displayed less glomerular damage compared to those given HP casein (Wesson et al. 2007).

Obesity also is a risk factor for increased blood pressure (Kang. 2013) which in turn is an important factor contributing to renal damage (Lindeman et al. 1984, Tozawa et al. 2003). Although HP diets have been shown to counteract the increase in blood pressure in overweight human subjects (Papakonstantinou et al. 2010), neither obesity nor protein level affected blood pressure in the present study. The lack of increased blood pressure in obese *fa/fa* Zucker rats compared to their lean counterparts has been reported previously (Rodriguez et al. 2012). However, dietary HP soy protein delayed the increase in blood pressure observed in the *faHM* and *lnNC* groups. This is consistent with other studies in animal models (Ibrahim et al. 2015, Palanisamy et al. 2010) and a meta-analysis of 25 randomized control trials that showed

significant reductions in blood pressure with soy protein consumption in both hypertensive and normotensive subjects (Dong et al. 2011). Given the current findings, however, treatment of high blood pressure with soy protein should be restricted to normal protein levels until the effects on renal damage in HP diets are further elucidated.

Some studies report a reduction in body weight with HP diets (Aparicio et al. 2013, Soenen et al. 2013, Noakes et al. 2005, Westerterp-Plantenga et al. 2012) while others find no such effect (Aldrich et al. 2011, Luscombe et al. 2003, Stern et al. 2004, Foster et al. 2003, Piatti et al. 1994, Noatsch et al. 2011). In the present study increasing the level of dietary casein did not alter body weight gain, but increasing the protein level using soy or mixed protein did result in higher body weights in these rats from week 5 onwards. This is in line with a cross-sectional analysis showing an increased risk of overweight and obesity in women consuming a greater proportion of energy from protein (Murtaugh et al. 2007). However, as previously reported in these rats, the HP diets resulted in higher lean mass (Wojcik et al. 2016), consistent with studies indicating that one of the benefits of HP diets is their effect on sparing fat free mass during weight loss (Farnsworth et al. 2003, Claessens et al. 2009).

The current study reveals that in the context of obesity and mild renal disease a HP mixed protein diet results in renal and glomerular enlargement but reduced serum MCP-1, and although it does not increase fibrosis, there is a small number of damaged glomeruli. On the other hand, the HP casein and soy protein diets do not affect the obesity induced renal and glomerular enlargement, but they display more renal fibrosis and do not lower serum MCP-1 levels. The HP soy protein diet also exhibits some glomerular damage, but it also delays the elevation in blood

pressure. Thus, inclusion of mixed protein sources in a HP diet may mitigate some of the detrimental effects of HP diets on renal damage observed with single protein sources. As diets normally contain mixed protein sources, the potential beneficial effects of HP diets in weight loss should therefore be considered in the context of the possible risks to renal health.

Acknowledgements

This study was supported by funding from CIHR MOP 230564.

Conflict-of-interest

Authors have no conflict of Interest to declare.

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Table 1: Diet formulations and macronutrient composition^{1,2}

Ingredients (g/100 g diet)		NC	HC	HS	HM
Protein	Casein (87% protein) ³	17.4	39.5	-	-
	Soy protein (87% protein) ³	-	-	40.6	10.0
	Wheat gluten (76% protein) ³	-	-	-	11.5
	Complete milk protein (89% protein) ³	-	-	-	10.0
	Egg white (82% protein) ³	-	-	-	10.6
Carbohydrates	Sucrose	10.0	10.0	10.0	10.0
	Cornstarch ⁴	39.6	17.4	18.3	15.7
	Dextrinized cornstarch	13.2	13.2	13.2	13.2
	Cellulose fiber	5.0	5.0	5.0	5.0
Fats	Soybean oil with TBHQ ⁵	9.8	9.6	8.2	9.3
AIN-93M mineral mix		3.5	3.5	3.5	3.5
AIN-93VX vitamin mix		1.0	1.0	1.0	1.0
Choline bitartrate		0.3	0.3	0.3	0.3
L-cystine		0.3	0.6	-	-
Energy Content	Protein (%energy)	15	35	35	35
	Carbohydrates (%energy)	64	44	44	44
	Fats (%energy)	21	21	21	21

¹Abbreviations: NC; normal protein casein; HC; high protein casein; HS; high protein soy; HM; high protein mixed; TBHQ; *tert*-Butylhydroquinone

²All ingredients purchased from Dyets Inc. (Bethlehem PA) except cornstarch which was purchased from Moonshiner Unlimited (Winnipeg MB).

³The protein sources were adjusted for the protein content, ensuring that the % energy from protein remained the same for the high protein diets and that all diets were isocaloric.

⁴Cornstarch was adjusted for the carbohydrate content of the protein sources, ensuring that the % energy from carbohydrates remained the same for the high protein diets and that all diets were isocaloric.

⁵Soybean oil was adjusted for the fat content in the protein sources, ensuring that the % energy from fat was the same for all four diets and that all diets were isocaloric

Table 2: Renal parameters

	lnNC	faNC	faHC	faHS	faHM
Average daily feed intake (g)	21±1.0	27±1.0 [⊕]	25±1.0 [⊕]	28±2.0 [⊕]	29±2.0 [⊕]
Kidney weight (g)	2.68 ± 0.10	4.47 ± 0.38 [⊕]	4.77 ± 0.36 [⊕]	5.95 ± 0.99 [⊕]	6.67 ± 0.99 ^{⊕⊘}
Kidney weight/body weight (g/100g)	0.49 ± 0.01	0.77 ± 0.12	0.67 ± 0.06	0.78 ± 0.13	0.89 ± 0.14 [⊕]
Fibrosis (pixels/field)	1056 ± 288	2164 ± 750	2848 ± 682 [⊕]	4571 ± 1633 [⊕]	1235 ± 304 [⊕]
Fibrosis (%)	0.27 ± 0.07	0.55 ± 0.19	0.73 ± 0.17 [⊕]	1.17 ± 0.42 [⊕]	0.32 ± 0.08 [⊕]
Mean glomerular volume (μm ³ x 10 ⁶)	0.71 ± 0.03	1.14 ± 0.07 [⊕]	1.06 ± 0.07 [⊕]	1.20 ± 0.08 [⊕]	1.44±0.10 ^{⊕⊘⊕⊕}
Damaged glomeruli/26 fields	0.0 ± 0.0	1.0 ± 0.4	1.2 ± 0.3	2.8 ± 1.0 [⊕]	3.0 ± 1.0 [⊕]
Serum MCP-1 (pg/ml)	126 ± 10	97 ± 10	109 ± 10	103 ± 12	70 ± 7 ^{⊕⊘⊕⊕}
Proteinuria (mg/ml)	6.7 ± 2.0	23.0 ± 3.7 [⊕]	21.4 ± 2.8 [⊕]	20.7 ± 3.0 [⊕]	23.7 ± 3.9 [⊕]
Serum creatinine (μg/ml)	3.7 ± 0.2	4.3 ± 0.5	4.8 ± 0.5	4.5 ± 0.3	6.0 ± 1.0 [⊕]

ln; lean rats; fa; obese rats; NC; normal protein casein; HC; high protein casein; HS; high protein soy; HM; high protein mixed

⊕ different than lnNC

⊘ different than faNC (obese rats only)

⊕ different than faHC (HP groups only)

⊕ faHM different than faHS

Figure 1: Body weight during the 12 week feeding period

- ⊕ all obese groups different than lnNC group for all time points between symbols
- ∅ different than faNC for all time points between symbols

Figure 2: Representative images of renal cortex

ln; lean rats; fa; obese rats; NC; normal protein casein; HC; high protein casein; HS; high protein soy; HM; high protein mixed

Figure 3: Mean arterial pressure

- ⊕ different than lnNC
- Φ faHM different than faHS

Draft

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Figure 2: Representative images of renal cortex
ln; lean rats; fa; obese rats; NC; normal protein casein; HC; high protein casein; HS; high protein soy; HM;
high protein mixed

Figure 2
193x104mm (300 x 300 DPI)

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