Published in final edited form as:
Exp Eye Res. 2014 November ; 128: 151–155. doi:10.1016/j.exer.2014.09.009.

7-ketocholesterol accumulates in ocular tissues as a consequence of aging and is present in high levels in drusen

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Abstract

We analyzed by LCMS lipid extracts of lens, retina (MNR) and RPE/Choroid (MPEC) from macaque monkeys 2–25 yr in age to determine their content of 7-ketocholesterol (7KCh) as function of age. In addition we also analyzed drusen capped with retinal pigment epithelium (RPE), RPE, and neural retina from human donors age 72–95 yr. The lowest 7KCh levels were found in monkey lens (<0.5 to 3.5 pmol 7KCh per nmol Ch), the second highest in MNR (1–15 pmol/nmol), and the highest in MPEC (1 to > 60 pmol/nmol). Despite individual variability all three tissues demonstrated a strong age-related increase. In older human donors 7KCh levels were significantly higher. The levels in human neural retina ranged from 8–20 pmol/nmol, similar to the oldest monkeys, but 7-KCh levels in RPE ranged from 200–17,000 pmol/nmol, and in RPE-capped drusen from 200–2,000 pmol/nmol, levels that would be lethal in most cultured cell systems. Most of the 7KCh is sequestered and not readily available to the surrounding tissue, based on published histochemical evidence that extracellular cholesterol (Ch) and cholesteryl fatty acid esters (CEs) are highly concentrated in Bruch's membrane and drusen. However, adjacent tissues, especially RPE but also choriocapillaris endothelium, could be chronically inflamed and in peril of receiving a lethal exposure. Implications for initiation and progression of age-related macular degeneration are discussed.

Keywords

7-ketocholesterol; monkey; retina; drusen; aging; human; cholesterol; lipoproteins; age-related macular degeneration

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Financial disclosure:
National Eye Institute Intramural Research program (IRR); NIH grant EY06109, Arnold and Mabel Beckman Initiative for Macular Research (D. Stambolian MD PhD; co-PI), unrestricted funds to the UAB Department of Ophthalmology from Research to Prevent Blindness, Inc., and from EyeSight Foundation of Alabama (CAC).

7-Ketocholesterol (7KCh) is an oxysterol with pro-inflammatory and cytotoxic properties that forms by the auto-oxidation of cholesterol (Ch) and cholesterol fatty acid esters (CEs). It is found throughout the cardiovascular system but in particularly high concentrations in oxidized extracellular lipoprotein deposits associated with atheromatous plaques (Ohtsuka et al., 2006). This oxysterol has been implicated in numerous age-related chronic diseases (Poli et al., 2013; Rodriguez and Larrayoz, 2010), including age-related macular degeneration (AMD). In human neurosensory retina and its supporting tissues, previous studies have shown that Ch and CEs accumulate in Bruch's membrane as a consequence of aging, attributed to lipoprotein particles of apparent intra-ocular origin (Curcio et al., 2011; Pikuleva and Curcio, 2014). This accumulation is more prominent in the macula than in the periphery. Ch and CEs are also abundant in drusen, extracellular lesions on Bruch's membrane that are pathognomonic for AMD. In monkey retina 7KCh has been found associated with lipoprotein deposits in Bruch's membrane and lumen of the choriocapillaris (Moreira et al., 2009). It can also be detected in the neural retina in the lumen of the capillary vessels (Moreira et al., 2009). These locations also correlate where apoB100 immunoreactivity has been reported for monkey retina (Tserentsoodol et al., 2006). This study had three goals: to determine if 7KCh increased with age in ocular tissues, to determine if 7KCh is detectable in drusen, and to determine if 7KCh levels could reach inflammatory and/or toxic values.

For this purpose monkey eyes (*Macaca mulatta*) were collected over several years from the Pathology Department of the Division of Veterinary Resources at the National Institutes of Health (NIH), after completion of approved NIH-wide institute protocols. Eyes were collected immediately after euthanasia. Ocular tissues were dissected, lyophilized, weighed and stored dry until analyses. The number of animals for which tissues were assayed were 60 (for lens, ages 4–18 yr), 101 (for neurosensory retina (NR) in its entirety, ages 2–25 yr), and 102 (for retinal pigment epithelium (RPE) –choroid, ages 2–25 yr). Dry weights were not available for all samples. All animal research was conducted in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in accordance with NIH guidelines.

Our study also utilized human tissues from 7 eyes of 7 non-diabetic Caucasian donors (6 females and 1 male), aged 84.4 yr ± 7.5 yr (mean ± standard deviation). Eyes were preserved in 2% glutaraldehyde and 1% paraformaldehyde in 0.1M phosphate buffer at 4°C < 6 hr and stored in 0.1M phosphate buffer at 4 °C until used (~1 yr). Fixed tissues are suitable for quantifying tissue lipids if properly validated (Ach et al., 2014; Carr et al., 1993; Gülcan et al., 1993; Wang et al., 2010). Globes were evaluated for chorioretinal pathology by internal inspection with a medical retina specialist, multimodal ex vivo imaging using color photography and spectral domain optical coherence tomography, and high-resolution histology, as described (Li et al., 2014). Eyes chosen for this study lacked chorioretinal pathology in the macula discernable by these methods. Drusen capped by RPE, sheets of adjoining RPE lacking drusen, and overlying neurosensory retina were physically isolated, as described (Rudolf et al., 2008; Wang et al., 2010). A large majority of these drusen would be considered hard, i.e., dome-shaped with solid homogenous interiors. RPE-capped drusen are mixed samples that are 37% by volume RPE and 63% druse (Wang et al., 2010).

Samples were collected in tissue triplets (RPE-capped drusen, RPE, and retina) from the same retinal quadrants, two triplets per donor, to account for storage effects, which would be the same within each eye. Extra-macular retina represents 90% of total retinal area and thus contains the numerical majority of drusen, even in AMD eyes (Lengyel et al., 2004). Extra-macular drusen are suitable for biochemical assay, because of their abundance and biomechanical tractability for isolation (Rudolf et al., 2008). The most affected quadrants varied among eyes. Samples were pooled across quadrants within an eye as necessary to ensure adequate sample size (Wang et al., 2010), resulting in a mean number of RPE-capped drusen of 88 (range, 41–194). Use of human tissues in this study conformed to Institutional Review Board regulations at University of Alabama at Birmingham.

Lipids were extracted from monkey and human samples using a combination of dry ethanol and methylene chloride. D7-Ch (25, 26, 26, 26, 27,27, 27, deuterated Ch, Cambridge Isotope Laboratories, Inc) was used as an internal standard. To dried monkey tissues, 100 nmol of D7-Ch with 2 ml of dry ethanol were added; the smaller human samples received 10 nmols. Following thorough homogenization, ethanol was removed, and residues were extracted with 1 ml of methylene chloride. The two fractions were pooled and dried. Pooled fractions were dissolved in 1 or 2 ml of N, N-dimethylformamide (DMF) and injected in the LCMS without further processing.

Analyses for 7KCh and Ch levels were performed using an Agilent 1200 Series HPLC equipped with an auto-sampler and a 1260 Quad pump as described (Moreira et al., 2009). Gradient conditions are described in Table 1. The column (Varian XRs Ultra 2.8 μm C8, 2×100 mm) was run at 0.15 ml/min, and the entire flow was directed into the mass spectrometer. The mass spectrometer was a Water/Micromass Q-TOF micro using atmospheric pressure ionization (APCi) in positive mode. The QTOF was controlled by Q-TOF Micro 4.1 software, as described (Moreira et al., 2009). Standards for 7KCh, Ch and various CEs were prepared at 0.1 mM. 7KCh forms a prominent M+H m/z 401 ion. Ch and CEs form an M-OH and M-fatty acid m/z 369 ion, respectively. Specific ions were quantified using peak area integration. The internal standard D7-Ch forms a M-OH ion of m/z 376 which can be independently quantified, although it co-elutes with Ch. No auto-oxidation of the D7-Ch to D7-7KCh was observed during extraction or sample processing. Any analyses where the detector was in “dead time” sample was re-injected ether-diluted or in a smaller volume. The QTOF is linear over 3 orders of magnitude, proving good range of detection in one analysis.

Results of assays for monkey tissues are shown in Fig. 1. The monkey lens contains significant amounts of Ch but no detectable CEs. The levels in monkey lens are extremely low ranging from < 0.5 to 3.5 pmol of 7KCh per nmol of Ch. However, there is a general increase with advancing age (Fig. 1). The MNR samples contained significantly higher levels of 7KCh than lens, ranging from 1 to 18 pmol/nmol. Despite individual variation, in 100 individual measurements in animals 2–25 yr of age, the age-related increase is still clear (Fig. 1). MNR contained barely detectable levels of CEs and these were not quantified.

The MPEC samples contain 3–4 times higher levels of 7KCh than the MNR, with levels ranging from 1– 68 pmol/nmol in 102 individual measurements of animals 2 to 25 yr of age

(Fig. 1C). Despite considerable variation, an age-related increase is clear. The MPEC samples contained significant levels of a variety of CEs but these were not quantified. In some samples 7KCh-fatty acid esters (7KFAEs) are detectable. These are intermediates between CEs and 7KCh. These esters are unstable especially when the fatty acids are unsaturated (containing double bonds). The most stable of these esters is 7K-18:0 (stearic acid) but the levels of 18:0 in the MPEC were below levels required for reliable quantification.

Results of assays of human tissues are shown in Table 2. There were 13 independent analyses from the 7 different individuals. Levels of 7KCh expressed as a dry weight percentage averaged 0.05% and 2.3% for Ch (w/w). In human neural retina 7KCh levels were similar that of the older monkeys, with values ranging from 8.5 to 21 pmol/nmol (mean, 16.5 pmol/nmol). RPE from the same human samples showed high and variable 7KCh levels, ranging from 200 to 17,000 pmol/nmol. Two individuals (72 and 81 yr) had measurements of over 3,000 pmol/nmol (Table 2). Mean 7KCh/Ch ratio for RPE samples including these two individuals is 2483 pmol/nmol and 458 pmol/nmol omitting them. The average dry weight % for 7KCh was 0.38% and 0.61% for Ch (w/w). The RPE also contained significant levels of CEs but no intermediate 7KFAEs. To our knowledge these high levels of 7KCh have been measured previously only in atheromatous plaques (Ohtsuka et al., 2006). Drusen samples from these same individuals also contained extremely high levels of 7KCh. These samples ranged from 200 to 2,000 pmol/nmol (mean, 734 pmol/nmol, Table 2). On average drusen contained 0.38% 7KCh and 0.49% Ch (w/w). There were also low but detectable levels of CEs in some drusen samples.

Our first major finding is a significant increase in 7KCh content, relative to Ch, in 3 monkey ocular tissues, especially in RPE-choroid, consistent with histochemical detection of Ch in monkey Bruch's membrane (Anderson et al., 2006). The use of lens as a within-eye control that also exhibits an age-related KCh increase on very low basal level strengthens this conclusion about other tissues in the same eyes. Our second major finding is high 7KCh content of human RPE and RPE-capped drusen, with lower levels in neurosensory retina, comparable to monkey, serving as an important within-eye controls.

7KCh has been historically challenging to assay, because it can form via Ch auto-oxidation during specimen storage and sample processing. We took several steps to ensure accuracy, including limited time in freezing, storage and processing of within-eye comparison tissues done in parallel, the lack of saponification to hydrolyze esters (Ohtsuka et al., 2006), and the use of a deuterated Ch internal standard. The low 7KCh level in lens and absent auto-oxidation of the standard suggest that these precautions were successful. Data should also be interpreted in light of differences in sample characteristics between monkey and human. Monkey eyes were freshly prepared whereas human samples were preserved. Monkey analyses used whole retina vs extra-macular pieces in human retina. Despite these differences, the combined dataset highlight the age-relatedness of 7KCh accumulation and its relationship with native Ch levels.

It is important to ask whether the measured levels of 7KCh have physiological relevance. The inflammatory dose for 7KCh on cultured ARPE19 cells is between 4–8 μM and the

lethal dose is 10–12 μM in 24 hr (Huang et al., 2012; Larrayoz et al., 2010). To put this into perspective a 1 mM solution of 7KCh (approximately 100x the lethal dose in cultured cells) is approximately 0.04% w/w. The tissue wet weight concentrations of 7KCh in the HPEC samples will be obviously lower due to the volume expansion in wet tissue. However, the 7KCh is not spread out throughout tissues but rather mostly localized in discrete deposits (Moreira et al., 2009). Therefore, levels in those deposits are likely reaching mM concentrations, of import because 7KCh will readily partition into the membranes of cells that contact these oxidized lipoprotein deposits. We have previously demonstrated that 7KCh can cause a massive inflammatory and angiogenic response *in vivo* by inserting 7KCh-containing implant in the anterior chamber of the rat eye (Amaral et al., 2013). This response includes aggregation of macrophages and VEGF induction, events that do not occur following implantation of native Ch. Thus 7KCh, along with linoleate hydroperoxide (Baba et al., 2010; Spaide et al., 1999), is a good candidate for instigating downstream sequelae of Ch-rich lipoprotein deposition in Bruch's membrane and drusen (Curcio et al., 2011; Spaide et al., 2003)

Our data from peripheral RPE-capped drusen and RPE have relevance to drusen with greater pathogenic significance for AMD progression in the macula. A major age-change in human Bruch's membrane is the accumulation of esterified and non-esterified Ch associated with lipoprotein particles (Curcio et al., 2011). Further the largest volumetric component of drusen (>40% of peripheral hard drusen) is chloroform-methanol extractable lipid, especially Ch and CE (Wang et al., 2010). Because drusen in human AMD macula are oily (Rudolf et al., 2008), their composition has been historically approached through techniques not requiring physical isolation such as transmission electron microscopy, lipid histochemistry, and immunohistochemistry (Curcio et al., 2005; Malek et al., 2003; Sarks et al., 1994). Biomechanical fragility in concert with lower apolipoprotein concentrations in soft drusen relative to peripheral hard drusen suggest that soft drusen are more lipid rich than peripheral hard drusen, with high Ch content. Thus we have reason to believe that 7KCh levels may be even higher in soft drusen, which are risk factors for inflammation and neovascularization, than those reported here for hard drusen.

Three points in the current data warrant future investigation in a larger sample of human tissues. First, histochemistry and thin layer chromatography indicate high content of CE in RPE-capped drusen that was not found in this study, although Ch is abundant by all methods. Dry weights of samples used in this study were small, and CE levels were near the limits of detectability. Further, ionization coefficients for CEs are low under APCI conditions that were optimized for 7 KK and Ch, as these were, and there was insufficient sample to try other methods suited for CE such as ESI. Second, the youngest of the human samples (72 and 81 yr) had by far the highest 7KCh levels while the oldest (92 and 95 yr) had the lowest, prompting the intriguing possibility of a relationship between relative resistance to 7KCh accumulation and longevity. Inspection of medical histories available to eye bank personnel indicated some donors with cardiovascular disease (e.g., stents, hyperlipidemia) without a systematic effect with aging. Third, whether 7KCh also accumulates in subretinal drusenoid deposit, a Ch-containing extracellular lesion that appears between photoreceptors and RPE and confers AMD risk independent of drusen

(Curcio et al., 2005; Oak et al., 2014; Zweifel et al., 2010a; Zweifel et al., 2010b), remains to be determined.

In summary, this study demonstrates that 7KCh accumulates in the neurosensory retina and its supporting tissues as a consequence of aging (Fig. 1). Both the aging effect and the absolute level of 7KCh are far more pronounced in the RPE/Choroid (Fig. 1, Table 1). This study also demonstrates that 7KCh can reach toxic levels, especially in humans (Table 2). However, it is likely that most of this 7KCh is not readily available to the surrounding tissues (it would be lethal) but is instead sequestered in drusen and/or complexed with other lipids and protein debris, which is known through histochemistry and direct assay to accumulate in and foul Bruch's membrane preferentially (Curcio et al., 2011; Pikuleva and Curcio, 2014). However, it is also likely that the RPE and choriocapillary endothelium near 7KCh-containing deposits are subjected to low but pro-inflammatory doses of 7KCh in aging. This will stress these tissues and cause a gradual loss of function that may explain some aspects of AMD pathogenesis.

Acknowledgments

We thank the personnel of the Alabama Eye Bank for timely recovery of donor eyes, eye donor families for their decision to donate, and Jeffrey D. Messinger, DC, for assistance with human tissue sample management.

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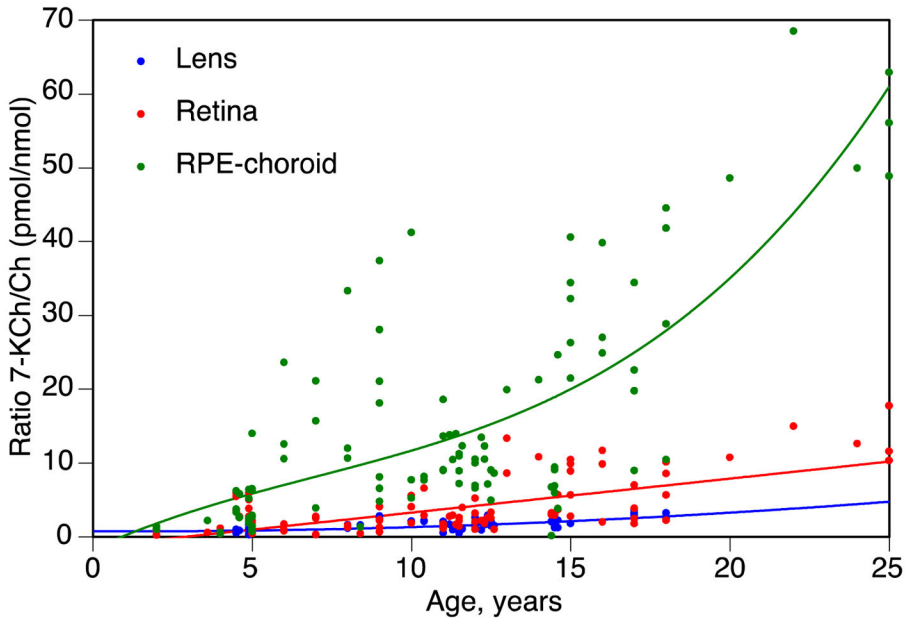

Fig. 1. 7KCh levels in monkey ocular tissues

Lens (n=60). Polynomial fit: $y = 6.93x^2 + -0.012x + 0.73$; $r^2 = 0.45$. Neural retina (n=100). Linear fit: $y = 0.46x + -1.33$; $r^2 = 0.44$. RPE/Choroid (n=102). Polynomial fit: $= 0.0056x^3 - 0.12x^2 + 1.96x - 1.65$; $r^2 = 0.61$. Fits to data used the lowest number of variables possible for each curve. They are descriptive only and not meant to capture underlying mechanism.

Table 1

Parameters for HPLC determination of 7-KetoCholesterol

Time	Solvent A	Solvent B	Solvent C	Solvent D
(min)	Water %	Acetonitrile %	Methanol %	MeOH/MeCl %
0	30	70	0	0
15	0	100	0	0
20	0	100	100	0
30	0	0	100	0
35	0	0	0	0
40	0	0	0	100
45	0	100	0	0
50	30	70	0	0

Table 2

Analysis of human neural retina (RET), RPE, and RPE-capped drusen (DRU).

	Age (yr)	7KCh (pmols)	Ch (nmols)	7K/Ch (pmols/nmols)	weight (μg)	Ch (nmols/μg)	7KCh (pmols/μg)	% 7KCh	% Ch
RET	72	2236.17	76.23	20.90	1108	0.07	2.02	0.08%	2.66%
RET	81	3315.48	190.19	17.43	3352	0.06	0.99	0.04%	2.19%
RET	81	1716.11	150.52	11.41	1994	0.08	0.86	0.03%	2.91%
RET	83	2457.66	247.92	11.31	5596	0.04	0.44	0.02%	1.71%
RET	83	2534.63	91.02	14.81	1162	0.08	2.18	0.09%	3.02%
RET	83	1864.07	97.85	19.04	1774	0.06	1.05	0.04%	2.13%
RET	83	1346.58	69.72	19.38	1374	0.05	0.98	0.04%	1.96%
RET	85	1866.26	101.45	10.89	1352	0.08	1.38	0.06%	2.90%
RET	85	3254.05	143.55	8.48	2350	0.06	1.38	0.06%	2.36%
RET	92	2931.46	135.26	21.64	1976	0.07	1.48	0.06%	2.64%
RET	92	1249.63	104.88	11.91					
RET	95	3932.20	292.33	24.23	6886	0.04	0.57	0.02%	1.64%
RET	95	3981.11	252.91	23.09	7156	0.04	0.56	0.02%	1.36%
			Mean	**16.5**			**Mean**	**0.05%**	**2.29%**
RPE	72	1830.40	0.11	16936.2	3182	0.003	1.64	0.07%	0.11%
RPE	72	2048.78	0.68	3023.0	56	0.012	36.61	1.46%	0.47%
RPE	81	460.43	0.08	5429.5	34	0.003	53.88	2.16%	0.12%
RPE	81	2300.54	0.48	4804.2	2602	0.001	0.67	0.03%	0.04%
RPE	83	1741.62	2.50	695.7	2216	0.000	1.04	0.04%	0.01%
RPE	83	2375.97	3.90	608.8	816	0.003	2.37	0.09%	0.11%
RPE	83	594.43	1.71	348.3	582	0.000	0.79	0.03%	0.01%
RPE	83	491.31	1.75	280.9	402	0.010	5.91	0.24%	0.38%
RPE	85	5207.47	8.92	583.7	NA				
RPE	85	1934.78	2.29	844.5	NA				
RPE	92	713.34	1.88	378.6	890	0.002	0.80	0.03%	0.08%
RPE	92	705.81	3.25	217.0	NA				
RPE	95	813.04	2.18	372.2	1289	0.002	0.63	0.03%	0.07%

	Age (yr)	7KCh (pmols)	Ch (nmols)	7K/Ch (pmols/nmols)	weight (μg)	Ch (nmols/μg)	7KCh (pmols/μg)	% 7KCh	% Ch
RPE	95	823.43	3.28	250.9	1663	0.002	0.50	0.02%	0.08%
			Mean	**2483.8**			**Mean**	**0.38%**	**0.61%**
DRU	72	2517.08	1.95	1291.0	126	0.047	22.31	0.89%	1.82%
DRU	81	1617.23	0.81	1996.5	120	0.010	8.77	0.35%	0.37%
DRU	81	1051.57	1.15	916.5	200	0.015	4.54	0.18%	0.56%
DRU	81	908.29	2.90	313.0	102	0.019	24.70	0.99%	0.74%
DRU	83	761.99	0.51	1481.4	188	0.003	4.06	0.16%	0.11%
DRU	83	1280.49	1.11	1149.6	152	0.005	10.65	0.43%	0.21%
DRU	83	948.05	1.63	581.5	162	0.007	7.91	0.32%	0.27%
DRU	83	1064.25	1.78	599.1	NA				
DRU	85	2809.04	5.94	472.6	NA				
DRU	92	479.84	1.54	311.5	NA				
DRU	92	616.47	1.21	511.3	NA				
DRU	92	725.14	3.99	181.8	NA				
DRU	95	532.10	2.10	253.5	614	0.003	0.87	0.03%	0.13%
DRU	95	575.71	2.52	228.5	524	0.005	1.10	0.04%	0.19%
			Mean	**734.9**			**Mean**	**0.38%**	**0.49%**

NOTE: Due to preparative losses one druse sample, only one retina sample, and 2 RPE samples remained out of the original tissue triplets (retina, RPE, druse) for the 72-year-old donor.